Glycoalkaloid Content and Starch Structure in *Solanum* Species and Interspecific Somatic Potato Hybrids

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ACADEMIC DISSERTATION

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ABSTRACT

When genome sections of wild *Solanum* species are bred into the cultivated potato (*S. tuberosum* L.) to obtain improved potato cultivars, the new cultivars must be evaluated for their beneficial and undesirable traits. Glycoalkaloids present in *Solanum* species are known for their toxic as well as for beneficial effects on mammals. On the other hand, glycoalkaloids in potato leaves provide natural protection against pests. Due to breeding, glycoalkaloid profile of the plant is affected. In addition, the starch properties in potato tubers can be affected as a result of breeding, because the crystalline properties are determined by the botanical source of the starch. Starch content and composition affect the texture of cooked and processed potatoes.

In order to determine glycoalkaloid contents in *Solanum* species, simultaneous separation of glycoalkaloids and aglycones using reversed-phase high-performance liquid chromatography (HPLC) was developed. A silica-based octadecyl chromatography column and acetonitrile-acidic triethylammoniumphosphate buffer as an elution solvent were used. Clean-up of foliage samples was improved using a silica-based strong cation exchanger instead of octadecyl phases in solid-phase extraction. Glycoalkaloids α-solanine and α-chaconine were detected in potato tubers of cvs. Satu and Sini. The total glycoalkaloid concentration of non-peeled and immature tubers was at an acceptable level (under 20 mg/100 g of FW) in the cv. Satu, whereas concentration in cv. Sini was 23 mg/100 g FW.

Solanum species (*S. tuberosum*, *S. brevidens*, *S. acaule*, and *S. commersonii*) and interspecific somatic hybrids (*brd* + *tbr*, *acl* + *tbr*, *cmm* + *tbr*) were analyzed for their glycoalkaloid contents using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). The LC-ESI-MS method proved to be well-suited to the analysis of complex mixtures of glycoalkaloids present in *Solanum* species and somatic hybrids. The tubers of *S. acaule* contained a high concentration of glycoalkaloids (48 mg/100 g FW). The concentrations in the tubers of the *brd* + *tbr* and *acl* + *tbr* hybrids remained under 20 mg/100 g FW. Glycoalkaloid concentration in the foliage of the *Solanum* species was between 110 mg and 890 mg/100 g FW. However, the concentration in the foliage of *S. acaule* was as low as 26 mg/100 g FW. The total concentrations of *brd* + *tbr*, *acl* + *tbr*, and *cmm* + *tbr* hybrid foliages were 88 mg, 180 mg, and 685 mg/100 g FW, respectively. Glycoalkaloids of both parental plants as well as new combinations of aglycones and saccharides were detected in somatic hybrids. The hybrids contained mainly spirosolanes, and glycoalkaloid structures having no 5,6-double bond in the aglycone. Based on these results, the glycoalkaloid profiles of the hybrids may represent a safer and more beneficial spectrum of glycoalkaloids than that found in currently cultivated varieties.

Starch nanostructure of three different cultivars (Satu, Saturna, and Lady Rosetta), a wild species *S. acaule*, and interspecific somatic hybrids were examined by wide-angle and small-angle X-ray scattering (WAXS, SAXS). For the first time, the measurements were conducted on fresh potato tuber samples. Crystallinity of starch, average crystallite size, and lamellar distance were determined from the X-ray patterns. No differences in the starch nanostructure between the three different cultivars were detected. However, tuber immaturity was detected by X-ray scattering methods when large numbers of immature and mature samples were measured and the results were compared. The present study shows that no significant changes occurred in the nanostructures of starches resulting from hybridizations of potato cultivars.

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LIST OF ORIGINAL PUBLICATIONS

I
Kuronen P, Väänänen T, Pehu E. 1999.
Reversed-phase high-performance liquid chromatographic separation and simultaneous profiling of steroidal glycoalkaloids and their aglycones. J Chromatogr A 863: 25-35.

II
Väänänen T, Kuronen P, Pehu E. 2000.
Comparison of commercial solid-phase extraction sorbents for the sample preparation of potato glycoalkaloids. J Chromatogr A 869: 301-305.

III
Turakainen M, Väänänen T, Anttila K, Ollilainen V, Hartikainen H, Seppänen M. 2004. Glycoalkaloid content in selenium-supplemented potatoes. J Agric Food Chem 52: 7139-7143.

IV
Väänänen T, Ikonen T, Jokela K, Serimaa R, Pietilä L, Pehu E. 2003.
X-ray Scattering study on potato (*Solanum tuberosum* L.) cultivars during winter storage. Carbohydrate Polym 54: 499-507.

V
Väänänen T, Ikonen T, Rokka V-M, Kuronen P, Serimaa R, Ollilainen V. 2005.
Influence of incorporated wild *Solanum* genomes on potato properties in terms of starch nanostructure and glycoalkaloid content. J Agric Food Chem 53: 5313-5325.
Correction. 2006. J Agric Food Chem 54: 4496-4497.

In addition, glycoalkaloids of *S. commersonii* and its somatic hybrid were analyzed by LC-ESI-MS, and glycoalkaloid contents of fresh and freeze dried tubers of *S. acaule* + *S. tuberosum* were compared. The data have been included in this thesis.

Author's contribution:

I Dr. Pirjo Kuronen offered the major contribution in planning the RP-HPLC-DAD conditions and columns for the simultaneous separation of glycoalkaloids and aglycones. The author of this thesis carried out the HPLC experiments and was partly planning the study. Professor Eija Pehu participated in writing the manuscript with Dr. Pirjo Kuronen, and the author.

II The author planned the study with Dr. Pirjo Kuronen. The author carried out the sample preparations and the HPLC experiments. The author had the main contribution to writing the manuscript. Dr. Pirjo Kuronen participated in writing the manuscript. Professor Eija Pehu commented and discussed the study, and offered the plant materials.

III The author was responsible for the sample preparation and the HPLC analysis of glycoalkaloids. The author, MSc. Anttila and MSc. Turakainen carried out the extraction procedure and the HPLC runs. MSc. Katja Anttila planned the experiment and was responsible for growing the plant material for the first experiment under the supervision of Doc. Seppänen and MSc. Turakainen. Doc. Ollilainen offered his guidance in the use of HPLC. In the second experiment, MSc. Turakainen planned the experiment and was responsible for growing the plant material. The author and MSc. Turakainen conducted glycoalkaloid analyses. MSc. Turakainen conducted Se analyses and the statistical analyses. The author and MSc. Turakainen were responsible writing the manuscript. Doc. Seppänen participated in writing the manuscript. Doc. Seppänen, Prof. Hartikainen and Doc. Ollilainen edited the manuscript.

IV The author was responsible for planning the study under the supervision by Dr. Ritva Serimaa. The plant materials were selected by the author, and Mrs. Leena Pietilä, who offered the potatoes. The author ran most of the X-ray experiments. Mr. Teemu Ikonen, and Ms. Kaija Jokela participated in running the X-ray experiments. Mr. Teemu Ikonen was responsible for all modelling and calculations of the X-ray masurements under supervision by Dr. Ritva Serimaa. The author, Mr. Teemu Ikonen, and Dr. Ritva Serimaa wrote the manuscript. Ms. Leena Pietilä, and Professor Eija Pehu took part in discussion of the results during the writing procedure.

V The author was responsible for planning the study together with Dr. Pirjo Kuronen, Dr. Ritva Serimaa, and Dr. Velimatti Ollilainen. Dr. Veli-Matti Rokka partly selected and offered the plant materials. The author carried out all the sample preparations and calculations of the results in the LC-MS study. The LC-MS experiments were run under the supervision by Dr. Velimatti Ollilainen. Teemu Ikonen carried out the X-ray experiments under the supervision by Dr. Ritva Serimaa. The author, Dr. Veli-Matti Rokka, Dr. Ritva Serimaa, and Dr. Velimatti Ollilainen participated in writing the manuscript.

LIST OF ABBREVIATIONS

acl	*Solanum acaule*
brd	*Solanum brevidens*
C_6	hexyl
C_8	octyl
C_{18}	octadecyl
CE	capillary electrophoresis
cmm	*Solanum commersonii*
CPB	Colorado potato beetle
cv.	cultivar, cultivated variety
DM	dry matter
DSC	differential scanning calorimetry
EI	electron ionization
ELISA	enzyme-linked immunosorbent assay
FAB	fast atom bombardment
FPIA	fluorescence polarization immunoassay
FTIR	Fourier-transform infrared spectroscopy
FW	fresh weight
FWHM	full-width at half maximum
gal	galactose
GC	gas chromatography
glc	glucose
GM	genetically modified
HLB	high lipophilic bond
HPLC	high-performance liquid chromatography
LC-ESI-MS	liquid chromatography electrospray ionization mass spectrometry
MALDI	matrix assisted laser desorption ionization
MS	mass spectrometry
NH_2	amino
NMR	nuclear magnetic resonance spectroscopy
PAD	pulsed amperometric detection
rha	rhamnose
RP-HPLC	reversed-phase high-performance liquid chromatography
RSD	relative standard deviation
SAXS	small angle X-ray scattering
SCX	strong cation exchanger
SEC	size exclusion chromatography
SPE	solid-phase extraction
tbr	*Solanum tuberosum*
TEAA	triethyl ammonium acetate
TEAP	triethyl ammonium phosphate
THF	tetrahydrofuran
TLC	thin-layer chromatography
TOF	time of flight
Tris	tris(hydroxymethyl)methylamine
USAXS	ultra small angle X-ray scattering
UV	ultra violet
WAXS	wide-angle X-ray scattering
xyl	xylose

CONTENTS

1. INTRODUCTION

The potato plant spread originally from the Andes of Peru to Europe with Spanish explorers in the 16th century, and then gradually to other parts of the world. The first introduction to potato farming and storing in Finland was written by Axel Laurell (Laurell, 1773), at a time when the potato was being established in South-Western Finland. By the 20th century, the potato had become one of the most important food staples as a source of carbohydrates. Potato production is about 320 million metric tons globally, of which about 66% are used as food, 12% as feed, and 10% as seed (FAOSTAT, 2006). The rest is used mainly in the starch industry, since increasing amounts of starch are now extracted from potato tubers and modified for further uses in processed foods and in non-foods, including the paper industry.

In everyday language the word "potato" refers to the tuber-bearing cultivated potato species *Solanum tuberosum* L., which belongs to the family Solanaceae, genus *Solanum*, section *Petota,* subsection *Potatoe* (Hawkes, 1990). Other well known cultivated species belonging to the same family are tomato (*Lycopersicon esculentum* or *S. lycopersicum*), egg-plant (*S. melongena*), and sweet and hot peppers (*Capsicum* spp.). In addition, the subsection *Potatoe* includes six other cultivated potato species as well as about 200 wild tuber-bearing potato species. The other subsection of the section Petota, *Estolonifera,* includes the non-tuberizing species. About 40 different cultivated varieties (cultivars, cvs.) of *S. tuberosum,* such as Van Gogh, Pito, and Satu, are sold in Finland.

The potato crop is threatened not only by such abiotic stresses as cold, frost, and drought, but also by viruses, bacterial diseases and pests. Improving the resistance and tolerance of the varieties to these threats is a great challenge for breeders, who are developing varieties for various purposes. Genome sections of wild *Solanum* species of the section *Petota*, or tomato, are bred into the cultivated potato on account of their beneficial traits: high level of resistance to potato viruses (traits which are derived from *Solanum* species *S. acaule, S. brevidens*), to nematodes (originated from *S. berthaultii*), to soft rot (*S. brevidens*), to frost or cold (*S. commersonii*), and to late blight resistance (*S. demissum)*. Due to limitations of using wild potatoes for sexual crosses with cultivated varieties, protoplast fusion can also be used to obtain interspecific wide hybrids (i.e. somatic hybrids) (Waara and Glimelius, 1995; Rokka et al., 1995; Rokka, 1998a, b; Pehu et al., 1989; Pehu et al., 1990a, b; Laurila, 2004). In order to reduce the proportion of the wild species genome from further generations, it was possible to fuse the interspecific hybrids with *S. tuberosum* to obtain "second-generation" somatic hybrids (Rokka et al., 2000), and thereby further improve the production of new cultivars for commercial use. Cultivars with high tuber yields of good quality are in high demand. Tuber quality is observed from appearance of fresh tubers, texture and color after cooking, nutritional value, and by content of undesirable components, such as nitrates, heavy metals, pesticides, and glycoalkaloids.

The glycoalkaloids in edible potato cultivars are continuously being studied. The natural presence of these toxic compounds in potato tubers has raised many questions about the safety of the potato for consumption. The safety limit for their concentration in edible cultivars is still under debate. On the other hand, because glycoalkaloids in potato leaves provide natural protection against pests the complete elimination of these compounds through breeding is not necessarily desirable. Because the glycoalkaloid content is dependent on the genetic background of the plant, the glycoalkaloid profiles are often considerably altered in potato hybrids. The toxic effects of alien glycoalkaloids, e.g. glycoalkaloids not present in commercial cultivars, must be evaluated before releasing new cultivars to the market (Kumlay et al., 2002). Interestingly, some of the alien glycoalkaloids may also have beneficial effects in human health. Currently, modern analytical methods have made it possible to determine the molecular structures of the glycoalkaloids and quantify their concentrations in potatoes. The latter are prerequisites for determine both the nature and quantities of glycoalkaloids in potatoes.

Starch content and composition affect the texture of cooked and processed potatoes. In addition, starch is used in foods to improve their texture. The behavior of starch in foods depends on the size and shape of the starch granules, which are detected by microscopy, and on their chemical and physical structure. For example, the degree of crystallinity and the crystalline structure of isolated starch can be measured by X-ray scattering methods. In addition, the gelation properties of starch–water suspensions are studied by differential scanning calorimetry. The crystalline properties are determined by the botanical source of the starch. Potato varieties containing starch with the desired properties have been generated by conventional breeding and modification by plant genetics. However, starch structure and properties of hybrid tubers have not yet been studied thoroughly.

The present thesis reviews the literature concerning glycoalkaloids and starch in edible potato cultivars, wild *Solanum* species, and interspecific hybrids. The experimental data and the results published in Papers I-V based on the thesis are attached. The signficance of the results are discussed. Based on the described observations, suggestions are offered for further needed research in each of these categories.

2. REVIEW OF THE LITERATURE

2.1 Glycoalkaloids in *Solanum* species

2.1.1 Structures

Solanum glycoalkaloids are secondary metabolites formed from the same precursors as steroids. Cholesterol, cholesteranol, and cycloartenol are alternative precursors for aglycone biosyntheses. The aglycones have the C_{27} steroid skeleton of cholestane. Nitrogen is adapted to cholesterol derivatives from amino acids glycine, arginine (Jadhav and Salunkhe, 1973; Jadhav et al., 1973), or L-arginine (Kaneko et al., 1976). The aglycones are divided into five different categories depending on their structure: solanidanes have fused indolizidine rings (Figure 1), spirosolanes have an oxa-azaspirodecane alkaloid portion (Figure 1), 22, 26-epiminocholestanes, α-epiminocyclohemiketals, and 3-aminospirostanes (structures not shown). Most of the glycoalkaloids found in *Solanum* species belong to solanidanes and spirosolanes (Figure 1).

The most common solanidanes are solanidine and its dihydrogenated form, demissidine. Kuhn et al. (1955 a, b) showed that "solanine", which had been discovered in potato in 1820, was a mixture of two different glycoalkaloids, α-solanine and α-chaconine, both with solanidine as an aglycone but bound to different sugar moieties. The glycoalkaloids α-solanine and α-chaconine are generally present in tandem in plants, especially in *S. tuberosum*. The 22R, 25R epimer of solanidine has been detected in tubers of *S. vernei*, but this finding has not been reported elsewhere (van Gelder and Scheffer, 1991). Tomatidine is the spirosolane aglycone of α-tomatine present in tomato (*Lycopersicon esculentum*) as well as in *S. brevidens*, and *S. acaule*, among other species. Dehydrotomatidine, which is 5,6-dehydrogenated tomatidine, is often detected in tandem with tomatidine structure, especially in tomato plants. The 22, 25-diastereoisomeric pairs of tomatidine and dehydrotomatidine, soladulcidine and solasodine respectively, have been detected in many *Solanum* species. It has been suggested that solanidanes and spirosolanes are formed through the same biosynthetic route until the last steps, where etioline is transformed to either type of aglycone (Kaneko et al., 1976; Petersen et al., 1993). Petersen et al. (1993) suggested that diastereoisomeric spirosolanes are formed through different pathways. Immediately after formation aglycones are glycosylated by glucosyltransferase enzymes (Stapleton et al., 1991).

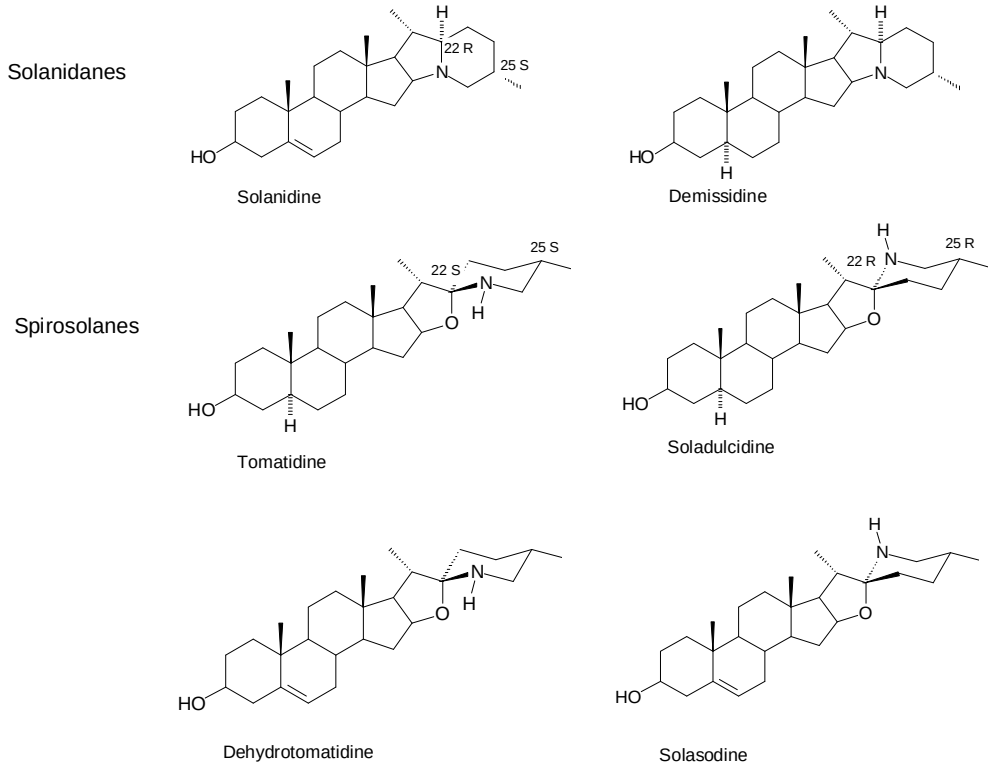


Figure 1. The structures of the most common *Solanum* aglycones.

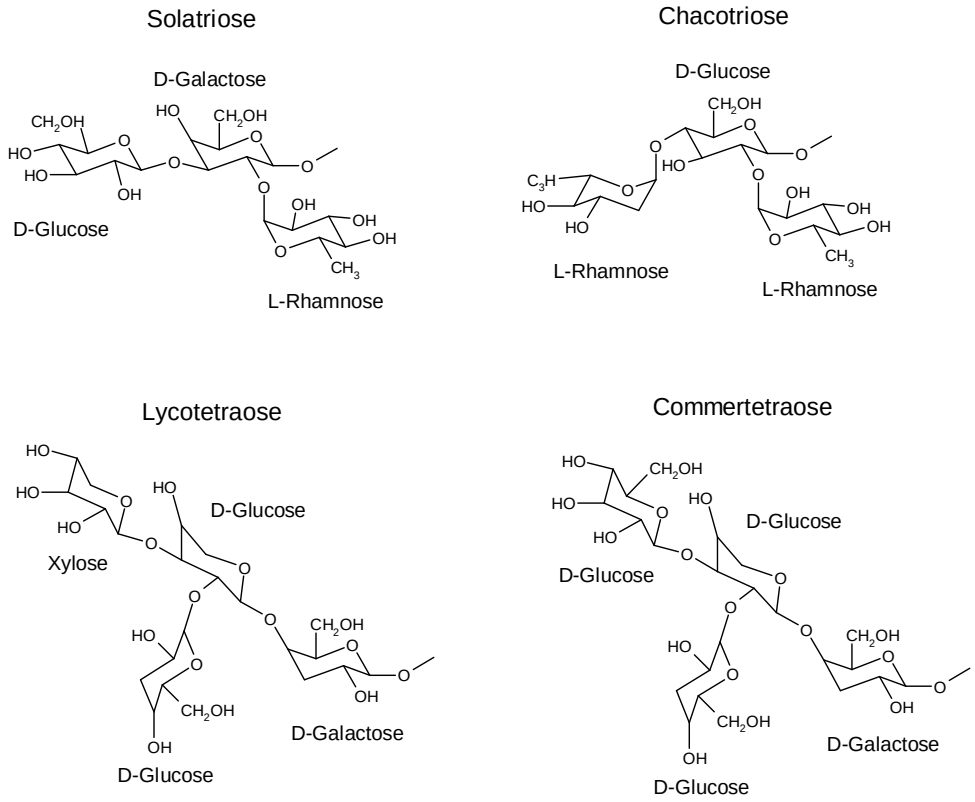
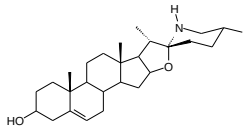
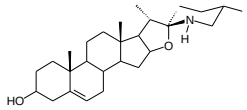


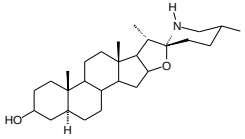
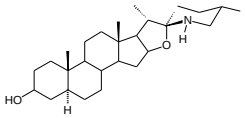
Figure 2. The common saccharides attached to the 3-hydroxy position of the aglycones.

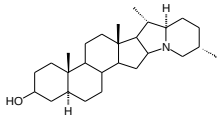
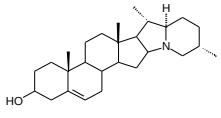
The saccharides attached to the 3-hydroxy position of aglycones consist of different combinations of D-glucose (Glc), D-galactose (Gal), D-xylose (Xyl), and L-rhamnose (Rha) in the form of tetra- or tri-saccharides. Minor amounts of aglycones attached to di- and monosaccharides, free aglycones, and their diene-products, being apparently artifacts, were detected in potato extracts (Osman and Sinden, 1977; Nikolic and Stankovic, 2003). The trisaccharide of α-solanine is called a solatriose, and that of α-chaconine, a chacotriose (Figure 2). A tetrasaccharide, lycotetraose, is present in α-tomatine but also in demissine, and soladulcine B (Table 1). Furthermore, another tetrasaccharide commertetraose is bound to solanidine, producing commersonine in *S. commersonii*, and to tomatidine, producing sisunine in *S. acaule* x *S. ajanhurii*. In fact, these four saccharides are the most common of the wide variety of *Solanum* glycoalkaloids. If the six major aglycones are combined with the four different saccharides, 24 different glycoalkaloids are obtained (Table 1). Most of them have been identified in *Solanum* plants of section *Petota*. However, some combinations have not been reported, e.g. tomatidine bound to solatriose, in any plants. In addition to the information given in Table 1, over 100 *Solanum* species have been analyzed for glycoalkaloids α-solanine, α-chaconine, α-tomatine, demissine, solamargine, and solasonine (Schreiber, 1968).

Furthermore, a group of glycoalkaloids called “leptines” consists of 23-hydroxy-solanidine or 23-acetoxysolanidine aglycones. Leptines I and II, as well as Leptinine I and II, are 23-hydroxy or 23-OAc analogs of α-solanine and α-chaconine, found in *S. chacoense* (Kuhn and Löw, 1961 a, b; Gregory et al., 1981; Sinden et al., 1986; Sanford et al., 1998). In addition to these glycoalkaloids, other rare solanidane or spirosolane glycosides were detected in *Solanum* plants (*S. suaveolens* by Ripperger and Porzel, 1997; *S. lyratum* by Lee et al., 1997; *S. arundo* by Fukuhara et al., 2004). Thus, a total of over 90 different glycoalkaloids have been characterized in over 300 *Solanum* species. Furthermore, novel or “alien” glycoalkaloid structures emerge as a result of genetic modification and hybridization of the *Solanum* plants.

Table 1. Solanidane and spirosolane glycoalkaloids found in *Solanum* plants.

Aglycone	Carbohydrate moiety	Compound trivial name	Solanum species	Reference et al.
Solasodine 	Lycotetraose	(not named)	*S. japonense* (taxonomy not known)	Murakami, 1984
	Solatriose	Solasonine	*S. berthaultii* *S. platanifolium* *S. ambosinum, S. multidissectum, S. spegazzinii,* *S. candolleanum, S. vernei, S. stoloniferum*	Gregory, 1981; Yencho, 1998. Puri, 1994 Deahl, 1993
	Chacotriose	Solamargine	*S. berthaultii* *S. paludosum* *S. ambosinum, S. multidissectum, S.spegazzini,* *S. bukasovii, S. candolleanum, S. santolallae,* *S. stoloniferum, S. vernei*	Gregory, 1981; Yencho, 1998 Valverde, 1993 Deahl, 1993
	Commertetraose	(not named)		not reported
Dehydrotomatidine 	Lycotetraose	Dehydrotomatine	*S. commersonii* *S. lycopersicum*	Carputo, 2003 Friedman, 1994
	Solatriose	α-Solamarine	*S. brachycarpum* *S. x curtilobum* (cultivated) *S. phureja x juzepczukii* *S. phureja* (cultivated) *S. candolleanum, S. medians, S. multiinterruptum*	Gregory, 1981 Osman, 1978 Osman, 1978; Schmiediche, 1980 Griffiths, 2000 Deahl, 1993
	Chacotriose	β-Solamarine	*S. trilobatum* *S. brachycarpum* *S. curtilobum* *S. x juzepczukii* (cultivated) *S. phureja* (cultivated)	Purushothaman, 1987 Gregory, 1981 Osman, 1978 Osman, 1978; Schmiediche, 1980 Griffiths, 2000
	Commertetraose	(not named)		not reported

Soladulcidine 	Lycotetraose	Soladulcine B	*S. lyratum*	Ye, 2001
	Solatriose	β-Soladulcine	*S. dulcamara*	Schreiber, 1958
	Chacotriose	Soladulcine A	*S. dulcamara*	Lee, 1994
	Commertetraose	(not named)		not reported
Tomatidine 	Lycotetraose	α-Tomatine	*S. brevidens* *S. acaule* *S. commersonii* *S. pinnatisectum* *S. polyadenium* *S. x juzepczukii* (cultivated) *S. demissum* *S. lycopersicum* *S. etuberosum, S. neocardenisii, S. okade, S. clarum* *S. oxycarpum, S. chomatophilum, S. jamesii,* *S. ochrantum*	Deahl, 1993 Osman, 1978 Vázquez, 1997 Gregory, 1981 Gregory, 1981; Deahl, 1993 Schmiediche, 1980 Schreiber, 1963a; Deahl, 1993 Friedman, 1994 Deahl, 1993
	Solatriose	(not named)		not reported
	Chacotriose	(not named)		not reported
	Commertetraose	Sisunine	*S. acaule x S. ajanhuiri*	Osman, 1986

Demissidine 	Lycotetraose	Demissine	*S. commersonii* *S. chacoense* *S. acaule* *S. demissum* *S. curtilobum* *S. x juzepczukii* (cultivated) *S. etuberosum, S. oxycarpum, S. chomatophilum, S. jamesii*	Vázquez, 1997; Osman, 1976; Deahl, 1993 Osman, 1976; Deahl, 1993 Gregory, 1981; Osman, 1978; Kozukue, 1999 Kuhn and Löw, 1947; Schreiber, 1963a; Deahl, 1993 Osman, 1978 Osman, 1978; Schmiediche, 1980 Deahl, 1993
	Solatriose	(not named)		not reported
	Chacotriose	(not named)		not reported
	Commertetraose	Commersonine	*S. commersonii* *S. chacoense*	Vázquez, 1997; Osman, 1976; Deahl, 1993 Osman, 1976; Deahl, 1993
Solanidine 	Lycotetraose	Dehydro-demissine	*S. commersonii*	Carputo, 2003; Vázquez, 1997
	Solatriose	α-Solanine	*S. tuberosum* *S. chacoense* *S x. ajanhuiri* (cultivated) , *S. stenotonum* (cultivated) *S. multidissectum, S. spegazzinii, S. bukasovii, S. candolleanum, S. medians, S. multiinterruptum, S. santolallae, S. stoloniferum, S. vernei*	Generally known Sanford, 1994; Deahl, 1993 Osman, 1978 Deahl, 1993
	Chacotriose	α-Chaconine	*S. tuberosum* *S. chacoense* *S.* x *ajanhuiri* (cultivated) *S. stenotonum* (cultivated), *S. multidissectum, S. vernei* *S. spegazzinii, S. bukasovii, S. candolleanum, S. medians, S. multiinterruptum, S. santolallae, S. stoloniferum*	Generally known Sanford, 1994; Deahl, 1993 Osman, 1978 Deahl, 1993
	Commertetraose	Dehydro-commersonine	*S. commersonii* *S. canasense*	Carputo, 2003; Vázquez, 1997; Gregory, 1981 Ramsay, 2004

2.1.2 Biological activity

Glycoalkaloids are potentially toxic compounds that have a role in the plant's protection system. Their toxicity is based their anti-cholinesterase activity on the central nervous system, to the disruption of cell membranes by complexation with membrane 3β-hydroxysterols, and to changes caused in active transport of ions through membranes, resulting in disorders in general body metabolism (Friedman et al., 1992a; Keukens et al., 1995; Blankemeyer et al., 1992; Blankemeyer et al., 1995). Toxic symptoms in humans include neurological and gastrointestinal disorders, such as vomiting, stomach pain, increased heart rate, and hallucinations.

Structure activity relationship and synergism. The amphiphilic nature of the glycoalkaloids contributes greatly to their anti-cholinesterase activity, aglycones as such being far less active than glycoalkaloids. Studies of the inhibition of acetylcholinesterase *in vitro* have revealed that α-solanine and α-chaconine have about equal effects (Roddick, 1989). Subsequent research has shown that although the presence of sugar moiety is obligatory for such activity, the aglycone structure determines the activity level (Roddick et al., 2001). However, differences in sugar moieties are thought to contribute to cell membrane disruption via sterol binding, α-tomatine being the most effective (Roddick, 1979; Keukens et al., 1992). Both α-solanine and α-chaconine appear to induce changes in sodium active transport and in membrane potential on frog skin (Blankemeyer et al., 1992; Blankemeyer et al., 1995). However, α-chaconine has been shown to be the most teratogenic compared to α-solanine and α-tomatine (Blankemeyer et al., 1997; Blankemeyer et al., 1998). Spirosolanes have shown lower activity than solanidanes, solanidine being the most teratogenic compared to solasodine and tomatidine (Gaffield and Keeler, 1996). The absolute configuration at C-22 and C-25 also determines the teratogenicity: synthetic 22S, 25R-epimer of solanidine appears to be more active than natural 22R, 25S -solanidine. In the same manner, 5,6-saturated 25R, 22R-solasodine is more active than 25S-22S-tomatidine. Furthermore, 5,6-unsaturation of the aglycone (solanidane or spirosolane) increases the activity.

Individual glycoalkaloids have been tested for their activity in biological systems. However, glycoalkaloids act synergistically, which means that the toxicities of individual glycoalkaloids do not predict the toxicities of mixtures of glycoalkaloids (Rayburn et al., 1994; Smith et al., 2001). In this context, the need for reliable techniques, such as LC-MS, for glycoalkaloid analyses cannot be underestimated.

Plant resistance to insects and diseases. According to Smith *et al.*, α-solanine and α-chaconine deters feeding of snails (Smith et al., 2001). Mixtures of solasonine and solamargine have been found to kill snails (Alzérreca and Hart, 1982), whereas tomatine has shown molluscicidal activity at low concentrations in tomato (Marston and Hostettmann, 1985). Resistance of hybrids to the Colorado potato beetle (CPB) seems to be connected to

foliage glycoalkaloid contents. High leptine (Rangarajan et al., 2000; Coombs et al., 2002), and high commersonine or dehydrocommersonine (Sinden et al., 1980) contents have been found to reduce feeding by CPB. Efforts to establish correlations between variations in the α-tomatine content of leaves and resistance to CPB in tomato plants have not been successful (Barbour and Kennedy, 1991). It has not been possible to correlate total glycoalkaloid contents of foliage and tubers in *S. berthaultii* or *S. tuberosum* with insect populations of potato leafhopper and fleabeetle (Tingey and Sinden, 1982). It has been concluded that tuber glycoalkaloid content may also not be responsible for late blight or soft rot resistance in potato (Sarquis et al., 2000; Andrivon et al., 2003; Andreu et al., 2001). However, alleopathic effects of solamargine and solasonine have been found to suppress seedling growth (Fukuhara et al., 1991).

Beneficial effects on human beings. In adition to their toxicity, glycoalkaloids also have beneficial properties. The role of α-tomatine in nutrition has been evaluated by Friedman (2002). In one of his finding that α-tomatine lowers plasma cholesterol and triglycerides (Friedman et al. 2000 a, b), Friedman challenged plant scientists to develop potatoes in which α-solanine and α-chaconine are replaced with α-tomatine. Other spirosolanes, such as solamargine, have anti-inflammatory effects and appear to act against herpes virus, and cancer cells (Cham, 2000; Kuo et al., 2000; Kuo and Lin, 2001; Ikeda et al., 2003; Lawson, 2003; Carter and Lake, 2004; Liu et al., 2004). In addition, α-chaconine is reported to inhibit human cancer cells cells (Lee et al., 2004).

2.1.3 Accumulation in cultivated potato

The cultivated potato *S. tuberosum* contains mainly α-solanine and α-chaconine in the ratio of 0.3 to 0.8 (α-solanine to α-chaconine) (Friedman, 2003). As α-chaconine is more toxic than α-solanine, a high ratio of α-solanine to α-chaconine is desired, the ratio being dependent on the cultivar. Minor amounts of solamarines were also found in the foliage and in aged as well as in wounded tubers (Shih and Kuc, 1974; Sinden and Sanford, 1981; Chivanov et al., 2001).

Plant parts above the soil, e.g. foliage, berries, and flowers, may contain hundreds of milligrams of glycoalkaloids per 100 g of FW. Concentrations in tubers are much lower. In Finland, as in many countries, the official recommended upper limit for total glycoalkaloid concentration in cultivated *S. tuberosum* tubers is 20 mg / 100 g FW (MTI, 2002). Because most of the glycoalkaloids in tubers are located in the skin or just under the skin (Sotelo and Serrano, 2000; Friedman, 2003), they can be removed to a large extent by peeling. Furthermore, the relative concentration of glycoalkaloids falls with increasing tuber size and maturation (Papathanasiou et al., 1999a; Papathanasiou et al., 1998). So-called “new potatoes” consumed especially in Scandinavia are generally harvested prior to maturity. They

are small in size, and enjoyed without peeling. As a result, they may be potentially toxic and cause acute symptoms, such as stomach and gastrointestinal disorders.
Glycoalkaloids accumulate in cytoplasm and in the vacuoles (Han et al., 1989) and are not diffused from the site where they accumulate (Roddick, 1982). However, information from the damaged part of a tuber or from leaves to tubers is transferred, leading to increased glycoalkaloid synthesis (van Gelder and Scheffer, 1991). Occasionally higher concentrations of glycoalkaloids have been found in commercially cultivated varieties (cv. Magnum Bonum) (Hellenäs et al., 1995a) as a result of unfavorable climatic conditions, such as drought or extreme temperatures (Papathanasiou et al., 1999b). As glycoalkaloid formation is enhanced by exposure to light (sun or artificial) (Percival et al., 1993; Percival et al., 1994; Percival et al., 1999; Lafta and Lorenzen, 2000), careless handling and storage after harvest can cause high glycoalkaloid concentrations in tubers (Griffiths et al., 1994; Griffiths et al., 1997; Coria et al., 1998). Greening of the tubers is a sign of exposure to light, and may thus be indicative of high glycoalkaloid concentration (Percival, 1999). However, the green color is chlorophyll, whose formation is independent of glycoalkaloid biosynthesis (Edwards et al., 1998; Kozukue et al., 2001). Potato tubers have been protected against light-induced glycoalkaloid formation by waxing with paraffin (Wu and Salunkhe, 1976), but this method is not in general use. Exposure to light can be avoided by storing potatoes in proper conditions after harvest. When potatoes are sold in bulk in the stores, exposure can be avoided by packing them in paper bags or in plastic bags that are opaque on one side.

Because it is a genetically controlled trait, glycoalkaloid content differs substantially from variety to variety (Hellenäs et al., 1995; Dimenstein et al., 1997; Papathanasiou et al., 1999b; Friedman, 2003). Some varieties have been withdrawn from the market because of their excessive glycoalkaloid accumulation (Blomberg and Penttilä, 2003). Thus, creating potato varieties with acceptable glycoalkaloid levels in tubers, but with adequate resistance against pests, is a challenge for breeders.

The effect of fertilization on glycoalkaloid contents has been studied very little. Selenium supplementation in soil has been shown to reduce total glycoalkaloid concentration in tubers (Mondy and Munshi, 1990; Munshi and Mondy, 1992). Glycoalkaloids in organically grown potatoes have also not attracted much interest. An exception is Kirchheim et al. (2004), who reported lower glycoalkaloid concentrations in organically grown tubers than in conventionally grownones. This result could not be confirmed by Wszelaki et al. (2005). However, the effect of soil and environmental conditions on glycoalkaloids is difficult to assess accurately, because of annual variation and the large number of potato varieties (Hajslova et al., 2005).

Genetically modified potatoes (transgenic potatoes) have been developed with improved resistance against viruses or with other desirable properties. The tuber peels of late blight resistant *S. tuberosum* cv. Desiree lines have been found to contain twice as many glycoalkaloids compared to control lines. However, the concentrations in flesh were nearly

the same or lower than in the conventional variety (Bianco et al., 2003). No significant increase in total glycoalkaloid contents was detected in potatoes resistant to CPB or virus Y (Rogan et al., 2000). However, the colorimetric method used by Rogan et al. did not detect differences between glycoalkaloid structures (Bergers, 1980). The α-solanine and α-chaconine contents, as determined by HPLC, were significantly lower in transgenic plants than in reference potatoes (Stobiecki et al., 2003; Zuk et al., 2003; Matthews et al., 2005).

Processed potato products also contain glycoalkaloids (Friedman and Dao, 1992b; Haase, 1998), because they are not thermally destroyed during cooking or frying (Bushway and Ponnampalam, 1981). Starch and potato protein extracts also contain glycoalkaloids (Saito et al., 1990; Alt, 2005). Feeding salmon with potato protein product with high levels of glycoalkaloids caused them to loose weight (Refstie and Tiekstra, 2003).

2.1.4 Influence of interspecific hybridization on glycoalkaloid profiles

The same glycoalkaloids as are present in parental lines are usually found in the foliage and tubers of the hybrids of *S. tuberosum* and wild species (Roddick and Melchers, 1985; Sanford et al., 1994; Laurila et al., 1996; Yencho et al., 1998; Kozukue et al., 1999) (Table 2). For example, hybrids of tomato and potato have been found to contain α-solanine, α-chaconine, and α-tomatine (Roddick and Melchers, 1985). Foliage of *S. berthaultii* and *S. tuberosum* hybrid contained solasodine based glycoalkaloids solasonine and solamargine (Yencho et al., 1998). Hybrids between *S. tuberosum* and *S. chacoense* contained α-solanine and α-chaconine in foliage, but α- and β-solamarines were also detected in parental *S. tuberosum* foliage (Sanford et al., 1994). Leptines present in *S. chacoense* were not found in tubers of any hybrid generation (Sanford et al., 1998). Backcrossing to *S. tuberosum*, however, reduced the total glycoalkaloid concentration of tubers from 52 mg in F2 hybrid to 27 mg in backcross (per 100 g FW).

In tubers of the somatic hybrids between dihaploid (2n=2x=24) *S. acaule* and tetraploid (2n=4x=48) *S. tuberosum*, the proportion of demissine and α-tomatine was very high, even as high as ~50% of the total glycoalkaloid concentration. The total glycoalkaloid concentration in the peels was reported to vary from 25 to 95 mg / 100 g FW (Kozukue et al., 1999). However, it was estimated that the content was nearly 20 mg/ 100 g FW in the whole tuber. Esposito et al. reported, that the total concentration of α-solanine and α-chaconine in the tubers of the backcross hybrids of *S. commersonii* and *S. tuberosum* was at the same level as in *S. tuberosum* controls, but lower than in their interspecific somatic hybrids (Esposito et al., 2002). According to subsequent research, glycoalkaloids dehydrodemissine, dehydrotomatine, and dehydrocommersonine from *S. commersonii* were also present in the tubers of their backcross progeny (Carputo et al., 2003). The glycoalkaloid profiles of leaf and tubers of the

backcross populations were similar, with their total glycoalkaloid concentrations varying between 11-32 mg / 100 g FW.

Aglycone analysis also provides information on the inheritance of glycoalkaloids from both parental lines as well as on the formation of new analogs. According to Laurila *et al*., foliage of somatic hybrids between *S. tuberosum* and *S. brevidens* contained solanidine and tomatidine inherited from parents, as well as a new aglycone demissidine. Repeated somatic hybridization to *S. tuberosum* resulted in the reduction of alien aglycones from 90% to 70%, the total aglycone concentration remaining at the same level as in *S. tuberosum* and the somatic hybrids (Laurila et al., 2001). Demissidine and tomatidine were also found in the tubers of hybrids between *S. circaefolium* and *S. tubersoum* (Mattheij et al., 1992), and solasodine aglycone was detected in the hybrids of *S. vernei* and *S. tuberosum* (van Gelder and Scheffer, 1991).

These observations indicate that the glycoalkaloid concentration seems to be nearly at or above the recommended limit of 20 mg/100 g FW in tubers of interspecific hybrids between *Solanum* species. Backcrosses and repeated hybridization seem to reduce glycoalkaloid concentrations as well as proportions of alien glycoalkaloids in tubers of further generations compared to the initial interspecific hybrids (Grassert and Lellbach, 1987). However, the glycoalkaloid profiles of backcross populations are still different from those of the cultivated *S. tuberosum* tubers containing normally only α-solanine and α-chaconine (Table 2). A tomatidine glycoalkaloid, sisunine, which is alien to both parental species, was found in sexual hybrids between *S. acaule* and *Solanum* x *ajanhuiri* (Osman et al., 1986).

Table 2. Aglycones and glycoalkaloids (other than α-solanine, and α-chaconine) found in the tubers of hybrids between *S. tuberosum* and other *Solanum* species, and in backcross populations with *S. tuberosum*.

Aglycone Glycoalkaloid	**Parental line species hybridized with S. tuberosum / hybrid type[1]**	**Approx. Proportion of total GAs**	**Reference (et al.)**
Solasodine	*S. vernei*	2%	van Gelder, 1991
Solasonine + Solamargine	*S. berthaultii* / BC	70%	Yencho, 1998
Dehydrotomatidine	*S. circaefolium* / SH	10%	Mattheij, 1992
Dehydrotomatine	*S. commersonii* / BC	40%	Carputo, 2003
Tomatidine	*S. circaefolium* / SH	30-50%	Mattheij, 1992
α-Tomatine	*S. acaule* / SH	30%	Kozukue, 1999
	tomato[2] / SH	60-70%	Roddick, 1985
Demissidine	*S. circaefolium* / SH	10-30%	Mattheij, 1992
Demissine	*S. acaule* / SH	40%	Kozukue, 1999
Solanidine	*(all)*		
Dehydrodemissine	*S. commersonii* / BC	60%	Carputo, 2003
Dehydrocommersonine	*S. commersonii* / BC	20%	Carputo, 2003

[1]SH = somatic hybrid, BC = backcross to *S. tuberosum*. [2]thickened stolons considered as tubers.

2.1.5 Analytical methods

2.1.5.1 Extraction and sample preparation

Glycoalkaloids are isolated from the plant material by solvent extraction preferably from dried plant material, or alternatively from fresh material. Drying of the plant material is conducted in the air or in the oven. However, freeze drying can also be used. Fresh materials are generally recommended to be transferred to ethanol to prevent enzymic browning (Bergers, 1980). According to Dao and Friedman (1996), the results of analyses of glycoalkaloids from freeze-dried leaf powders were more reproducible than those from fresh leaves. The main advantages of freeze drying in glycoalkaloid analysis are (1) reduced enzyme-catalyzed, wound-induced, and moisture-dependent compositional changes which may affect glycoalkaloid content, and (2) transportation of samples for analysis at different times and by different investigators is facilitated (Dao and Friedman, 1996). No degradation of glycoalkaloids in freeze drying has been reported.

Glycoalkaloids are soluble in acidic aqueous solutions and polar organic solvents, including acetonitrile, methanol, ethanol, and propanol. In most cases glycoalkaloid extraction from plant materials is carried out with dilute acetic acid (1-5%), a nontoxic and inexpensive solvent. Organic solvents such as methanol (Fitzpatrick and Osman, 1974) and methanol mixed with chloroform (Wang et al., 1972) have been selected especially for fresh samples. It has been reported, however, that aqueous solvents are more efficient for dried materials than non-aqueous (Bushway et al., 1986; Friedman and McDonald, 1995). Combinations of different solvents have been prepared to achieve effective extraction of amphiphilic glycoalkaloids, and they have been thoroughly reviewed (Friedman and McDonald, 1997, 1999). The possible hydrolysis of glycoalkaloids is avoided by using dilute weak acids or organic solvents at room temperature. However, aqueous methanol containing 1% hydrochloric acid has also been used (Kozukue et al., 1999; Stobiecki et al., 2003). Bisulfite is sometimes added to prevent oxidation of the extract (Hellenäs, 1986; Edwards and Cobb, 1996). Different procedures are used if glycoalkaloids are hydrolyzed in the sample preparation step. For example, two-phase hydrolysis and concomitant hydrolysis-extraction procedures have been succesfully applied to glycoalkaloid mixtures (van Gelder, 1984; Laurila et al., 1996; Weissenberg, 2001).

After extraction, the glycoalkaloids are either precipitated with ammonia (Fitzpatrick and Osman, 1974; Bushway et al., 1979; Dao and Friedman, 1996; Sotelo and Serrano, 2001; Kozukue and Friedman, 2003), or isolated with solid-phase extraction (SPE) (Carman et al., 1986; Bushway et al., 1986; Jonker et al., 1992; Friedman et al., 1994; Abell and Sporns, 1996; Edwards and Cobb, 1996; Friedman et al., 1998a; Esposito et al., 2002), or a combination of these methods. After precipitation with ammonia at pH ~10, the dried glycoalkaloid precipitation is dissolved in methanol or some other suitable solvent, or further extracted with butanol (Sotelo and Serrano, 2000). Because α-chaconine was present in the

liquid phase at high pH, the precipitation method may not give quantitative results (Gregory et al., 1981). Low recovery with precipitation supported using the SPE method for α-tomatine purification (Friedman et al., 1994). In general SPE procedures, the glycoalkaloids are retained in a sorbent in the first step, impurities are washed-off the phase in the second step, and finally glycoalkaloids are eluted with a suitable solvent. Various SPE materials and ready-to-use cartridges are commercially available. The most frequently used sorbent type is the octadecyl phase (C_{18}). Of the commercial cartridges, Sep-Pak C_{18} by Waters has been used most frequently (Bushway et al., 1986; Carman et al., 1986; Hellenäs, 1986; Saito et al., 1990; Ferreira et al., 1993; Abell and Sporns, 1996; Edwards and Cobb, 1996; Hellenäs and Branzell, 1997; Sanford et al., 1998; Simonovska and Vovk, 2000; Esposito et al., 2002; Carputo et al., 2003). Only aqueous extracts can be loaded to C_{18} SPE, because glycoalkaloids are not retained in the hydrophobic phase in organic solvent. The use of heptane or pentane sulfonic acid for ion-pair reagent enhanced adsorption of glycoalkaloids on non-polar SPE sorbent (Carman et al., 1986; Bushway et al., 1986; Edwards and Cobb, 1996). Amino, cyano (Jonker et al., 1992), and other phases (Alt, 2005) are also used in preparing glycoalkaloid samples. The SPE method is generally tested by spiking an extract with known amounts of glycoalkaloid standards. In most of the studies no calculation method has been reported, but generally the recovery of spiked glycoalkaloids has been calculated with an equation (Eldridge and Hockridge, 1983; Kozukue et al., 2004):

$$\text{Recovery} = \frac{a}{b+c} \times 100\%$$

where a = total concentration of glycoalkaloid in the spiked sample, b = concentration of endogenous glycoalkaloid, c = spiked amount (calculated).
IUPAC recommends the following equation (Burns et al., 2002):

$$\text{Recovery} = \frac{a-b}{c} \times 100\%$$

2.1.5.2 Chromatography

High-performance liquid chromatography. The most preferred technique for glycoalkaloid separations is reversed-phase high-performance liquid chromatography (RP-HPLC), in which glycoalkaloids are eluted from nonpolar stationary phases, e.g. silica-based octadecyl (C_{18}) phase columns, with aqueous elution solvents. Phases with embedded polar groups, e.g Supelcosil ABZ, have also been applied to glycoalkaloid analysis (Friedman and McDonald, 1997). The first HPLC separations of glycoalkaloids were carried out with tetrahydrofuran-water-acetonitrile mobile phase and a C_{18}, amino, or carbohydrate column (Bushway et al., 1979; Bushway and Ponnampalam, 1981; Bushway et al., 1986).

Table 3. Columns and elution solvents used in glycoalkaloid separations by high-performance liquid chromatography.

Column	Elution solvents	GAs separated	Reference (et al.)
Radially packed silicic acid	acetonitrile-water-ethanolamine	partial hydrolysis products of α-solanine, α-chaconine; solanidine, solanidiene	Morris and Lee 1981
Radially packed silicic acid	acetonitrile-phosphoric acid-ethanolamine pH 4.0	α-solanine, α-chaconine, α-solasonine, α-solamargine, β-solamargine	Eldridge, 1983
Shandon ODS-hypersil	acetonitrile-water-ethanolamine	α-solanine, α-chaconine	Hellenäs, 1986
Altex/Beckman C_8	acetonitrile-ammonium phosphate	α-solanine, α-chaconine	Carman, 1986
Chromospher C_{18}	acetonitrile-diammonium phosphate- triethylamine	solasonine, solamargine	Magrini, 1989
Spheri-5 C_8 and C_{18}	acetonitrile-Tris-HCl pH 7.4	α-solanine, α-chaconine, α-solasonine, α-solamargine	Jonker, 1992
Ultremex C_6	acetonitrile-methanol-ammonium phosphate pH 3.5	α-solanine, α-tomatine, commersonine	Bushway, 1994
Supelcosil C_{18}-DB	acetonitrile-sodium phosphate-dibutylamine pH 3	solanidine, dehydrotomatidine, tomatidine, solasodine	Friedman, 1994
Resolve C_{18} Supelcosil C_{18}	acetonitrile-ammonium phosphate pH 3.5	partial hydrolysis products of α-solanine and α-chaconine; solanidine, solasodine	Friedman and Levin, 1992; Friedman and McDonald,1995
Techsphere 80 C_8, C_{18} Zorbax C_8	acetonitrile-Tris-HCl pH 7.8	α-solanine, α-chaconine	Edwards and Cobb, 1996
Hypersil ODS	acetonitrile-potassium phosphate pH 7.60	α-solanine, α-chaconine	Hellenäs, 1997
Supelcosil LC-ABZ	acetonitrile-ammonium phosphate pH 3	α-tomatine, dehydrotomatine	Friedman 1997
μBonda-Pak C_{18}	acetonitrile-ammonium phosphate pH 6.5	α-solanine, α-chaconine	Sotelo, 2000
NH_2 and carbohydrate	THF-acetonitrile-water	α-solanine, α-chaconine	Bushway, 1981
Nucleosil 5-NH_2	acetonitrile-potassium phosphate pH 6	α-solanine, α-chaconine	Saito, 1990
Nucleosil NH_2	THF-acetonitrile-potassium phosphate	α-solanine, α-chaconine, α-tomatine, demissine	Kozukue, 1999
Nucleosil NH_2	acetonitrile-potassium phosphate pH 4.7-7.0	α-solanine, α-chaconine	Friedman, 2003
Reprosil-Pur NH_2	acetonitrile-potassium phosphate pH 6	α-solanine, α-chaconine	Alt, 2005

In most instances today, organic solvents are mixed with an aqueous buffer solution to elute glycoalkaloids from octyl (C_8) or C_{18} phase columns (Table 3). The separation of α-solanine and α-chaconine has been conducted with methanol-aqueous buffer elution (Crabbe and Fryer, 1980), and acetonitrile-ammonium phosphate elution (Carman et al., 1986), later applied to α-solanine and α-chaconine at different pH values (Friedman and Levin, 1992; Dao and Friedman, 1996; Sotelo and Serrano, 2000). This method has proved to be suitable also for hydrolytic products of α-chaconine (Friedman and McDonald, 1995) as well as for α-tomatine and dehydrotomatine separation at pH 3 (Friedman and Levin, 1998). Instead of ammonium phosphate, potassium phosphate at pH 7.6 was used with a C_{18} column (Hellenäs and Branzell, 1997). The glycoalkaloids α-solasonine, α-solamargine, α-solanine, and α-chaconine were separated with an octyl (C_8) column and acetonitrile-Tris-HCl at pH 7.8 (Jonker et al., 1992). Commersonine, tomatine, and α-solanine were separated with a hexyl (C_6) column and acetonitrile-methanol-ammonium phosphate buffer at pH 3.5 (Bushway et al., 1994). Generally, RP columns, being stable at low but not at high pH conditions, are operated at pH 2-8. Amphiphilic glycoalkaloids interact with non-polar stationary phases through Van der Waals forces. In addition, approximately at pH 4-9, ionic interaction between residual silanols and ionized glycoalkaloids takes place and may cause peak tailing (Edwards and Cobb, 1996). In acidic conditions, pH 1-3, ionic interaction between the solute and the silanols is suppressed and the retention times are reduced.

Similar solvent systems are used with aminopropyl phases. Separations on amino columns are aided by adding of phosphate buffer (Saito et al., 1990; Kozukue et al., 1999; Friedman, 2003; Kozukue and Friedman, 2003; Alt, 2005; Hajslova et al., 2005). No effect on retention times of α-solanine and α-chaconine determined with amino columns was detected with the variation of pH 4.7-7.0 (Friedman et al., 2003). However, the separation and the retention times varied with the buffer concentration of 1-40 mM, 20 mM being an optimal concentration.

In addition to pH adjustment, elution solvents have been modified by adding ethanolamine to improve the chromatography (Morris and Lee, 1981; Hellenäs, 1986). With the modified method of Morris and Lee (1981), solamargines, solasonine, α-solanine, and α-chaconine were separated in one run at pH 4.0 (Eldridge and Hockridge, 1983). Solasonine and solamargine were separated with the aid of triethylamine in acteonitrile-phosphate buffer (Magrini et al., 1989), whereas dibutylamine was used as in aglycone analysis (Friedman et al., 1994). Better peak shape can be achieved with these modifications because alkylammonium ions block ionized silanol groups by ionic interaction. Furthermore, heptanesulphonic acid was added to mobile phase in ion-pair chromatography of glycoalkaloids (Sanford et al., 1998).

Glycoalkaloid separations with HPLC are seldom run using a solvent gradient (Stobiecki et al., 2003). However, the runs are often conducted at elevated temperatures up to 50 ºC to improve separation of glycoalkaloids and to achieve good run repeatability (Saito et al., 1990; Jonker et al., 1992; Hellenäs and Branzell, 1997; Friedman et al., 2003; Alt, 2005). Column sorbents, C_{18} or amino, have enormous differences depending on the manufacturer. Repeatable runs as well as optimal retention times, separation, and peak shapes can be achieved using a wide range of HPLC methods (Edwards and Cobb, 1996; Friedman et al., 2003). However, aglycones and glycoalkaloids have previouslyu not been eluted in a single HPLC run.

Glycoalkaloids lack a strong UV chromophore, and only slight differences in the UV spectra recorded at 200 - 208 nm are seen between 5,6-double bond glycoalkaloids and the glycoalkaloids lacking it. Liquid chromatography with pulsed amperometric detection (PAD) is commonly used in carbohydrate analysis. It was applied to glycoalkaloids, PAD having a higher sensitivity than UV detection (Friedman et al., 1994). However, PAD was applied only for analytical LC because of possible permanent changes in the chemical structures of glycoalkaloids in the PAD system (Friedman and Levin, 1998). Recently, chemiluminescence has also been used in glycoalkaloid detection (Kodamatani et al., 2005).

Gas chromatography **(GC)** is applied to aglycone analysis after hydrolysis of glycoalkaloids (Osman and Sinden, 1977; Coxon et al., 1979; King, 1980; Bushway et al., 1984; van Gelder et al., 1988) or to sugars after derivatization (Herb et al., 1975; Osman et al., 1978; Gregory et al., 1981; Quidde et al., 1998). Good separation of a complex mixture of aglycones was obtained using capillary GC with a combination of a flame ionization detector (FID) and nitrogen source detector (NPD) (van Gelder et al., 1988b). More recently developed GC-MS methods have replaced other GC detection systems in glycoalkaloid analysis. Diastereoisomers solasodine and dehydrotomatidine, and soladulcidine and tomatidine if present, were separated as silylated forms by GC-MS (Laurila et al., 1999). Aglycones from potato hybrids were successfully quantified using this method (Laurila et al., 2001). Despite of good separation of aglycones, the need to hydrolyze glycoalkaloids is an outstanding disadvantage of GC methods, because information about glycoalkaloid diversity in *Solanum* plants is lost. However, profiling aglycones gives a clue to the complexity of glycoalkaloid mixtures extracted from plants, and it can be used in glycoalkaloid analysis.

Thin-layer chromatography. Glycoalkaloids or aglycones have been separated on thin-layer chromatography (TLC) silica plates (Schreiber et al., 1963b; Coxon, 1984), developed with 95% methanol or ethanol (Roddick and Melchers, 1985), methanol-chloroform/dichloromethane-aqueous ammonia (Bushway and Ponnampalam, 1981; Ferreira et al., 1993; Bodart et al., 2000; Nikolic and Stankovic, 2003), and acetic acid-ethyl acetate-methanol-water (Wang et al., 1972; Lairini and Ruiz-Rubio, 1997; Quidde et al., 1998). Partial hydrolysis products of α-solanine and α-chaconine were separated (Filadelfi and Zitnak, 1983). Tomatidenol, soladulcidine, and solasodine were separated on silica plates

impregnated with silver nitrate (Rozumek, 1969). Reversed-phase TLC plates (RP-C_8) were used with methanol-aqueous ammonium acetate elution to separate solamargine, xylosylsolamargine, solasonine, and four other glycoalkaloids (Ripperger and Porzel, 1997). Glycoalkaloids are visualized with Dragendorff's reagent (Roddick and Melcehrs, 1985; Ferreira et al., 1993), iodine vapor (Weissenberg, 2001), sulphuric acid and heat (Lairini and Ruiz-Rubio, 1997; Quidde et al., 1998; Nikolic and Stankovic, 2003), fluorodensitometry (Jellema et al., 1981), Carr-Price reagent (Ferreira et al., 1993) combined with densitometry (Bodart et al., 2000). TLC is primarily used for qualitative and preparative purposes, but a quantitative method has also been developed (Jellema et al., 1981; Ferreira et al., 1993; Simonovska and Vovk, 2000).

2.1.5.3 Mass spectrometry

The molecular mass of a glycoalkaloid can be determined by mass spectrometry. Furthermore, fragmentation of an aglycone or cleavage of saccharides from glycoalkaloids gives information about the molecular structure. The first mass spectra of aglycones were obtained by electron ionization (EI) (Budzikiewicz, 1964; Quyen et al., 1995). Since then, ionization methods applied also to intact glycoalkaloids have included fast atom bombardment (FAB) (Price et al., 1985; Self, 1987), matrix-assisted laser desorption/ionization (MALDI) coupled with time-of-flight mass spectrometry (TOF-MS) (Abell and Sporns, 1996; Quidde et al., 1998; Driedger and Sporns, 1999; Chivanov et al., 2001; Carputo et al., 2003), and electrospray ionization (ESI) coupled with a single quadrupole mass analyzer (Rodriguez-Saona et al., 1999; Esposito et al., 2002; Matsuda et al., 2004).

Carbohydrate sequences and linkage sites in the glycoalkaloids α-solanine, α-chaconine, demissine, α-tomatine, and α-solasonine have been determined from mass fragments produced by collision-induced dissociation (CID) in a four-sector tandem spectrometer (Evans et al., 1993; Chen et al., 1994; Claeys et al., 1996). The fragmentation of α-solanine, α-chaconine, and α-tomatine was carried out by multistage tandem mass spectrometry (MSn) with an ion trap (Bianco et al., 2002; Bianco et al., 2003). Tandem mass spectrometry with triple quadrupole was applied to α-solanine, α-chaconine, α-solamargine, and α-solasonine (Stobiecki et al., 2003).

Coupling a mass spectrometer to LC (LC-MS) (Stobiecki et al., 2003; Matsuda et al., 2004; Zywicki et al., 2005; Cataldi et al., 2005), to GC (GC-MS) (Van Gelder et al., 1989; Rick et al., 1994; Friedman et al., 1998a; Laurila et al., 1999; Nikolic and Stankovic, 2003), or to capillary electrophoresis (CE-MS) (Driedger et al., 2000a, b; Bianco et al., 2002; Bianco et al., 2003) has enabled separation and characterization of glycoalkaloids with the same molecular masses. Although LC-MS is a powerful tool in qualitative analysis of intact

glycoalkaloids, it has only infrequently been applied to quantitation of glycoalkaloids from plant materials (Bianco et al., 2003; Matsuda et al. 2004; Zywicki et al., 2005).

2.1.5.4 Immunoassay

In immunoassays, an antibody specific for a compound or a class of compounds binds to a compound or to a conjugate of a compound of interest. Immunochemical methods are distinguished by labelling the compound. Enzyme-linked immunosorbent assay (ELISA) has been used successfully for analysis of total glycoalkaloids (Morgan et al., 1983; Morgan et al., 1985; Ward et al., 1988; Friedman et al., 1998b), aglycones (Plhak and Sporns, 1994), tomatine (Driedger et al., 2000), solamargine (Tanaka et al., 1997; Putalun et al., 2000), and potato and tomato glycoalkaloids (Stanker et al., 1994). Hellenäs et al. (1986) found good agreement between HPLC and ELISA determinations. ELISA methods have advantages over alternative procedures: sample preparation is minimized, they are easy to use and no expensive equipment is required. ELISA kits can also be used in the field and they are appropriate for breeders. However, ELISA cannot be used in the analysis of complex mixtures of glycoalkaloids and in structure determination, where chromatography and structure determination are required. Faster and more precise than ELISA, solution-phase immunoassays of glycoalkaloids based on quantification by capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection has been developed (Driedger et al., 2000). In addition, fluorescence polarization immunoassay FPIA (Thomson and Sporns, 1995), liposome immunomigration (Glorio-Paulet and Durst, 2000), and flow-injection liposome immunoanalysis system (Kim et al., 2003) have been applied to glycoalkaloids.

2.1.5.5 Other methods

Nuclear magnetic resonance spectroscopy. The complete structure elucidation of isolated and purified aglycones and glycoalkaloids has been carried out with nuclear magnetic resonance spectroscopy (NMR). Chemical shifts (^{13}C) of solanidine, demissidine, solasodine, soladulcidine, and tomatidine (Radeglia et al., 1977) as well as of solasonine and α-tomatine (Weston et al., 1977) have been measured. The following glycoalkaloids isolated from *Solanum* plants were identified with the aid of two dimensional ^{1}H- and ^{13}C-NMR methods: solamargine (Valverde et al., 1993); solasonine, and solamargine from *S. platanifolium* (Puri et al., 1994); commersonine, dehydrocommersonine, demissine, dehydrodemissine, and tomatine from *S. commersonii* (Vázquez et al., 1997), from *S. amygdafolium* (Vazquez et al., 1999); soladulcine B from *S. lyratum* (Ye et al., 2001), from *S. arundo* (Fukuhara et al., 2004). NMR was also used to determine the structure of dehydrotomatine (Ono et al., 1997).

Single crystal X-ray diffraction. X-ray diffraction data have been obtained from solanidine, demissidine, and tomatidine in the form of N-hydrobromide or hydroiodide (Höhne et al.,

1966, 1967; Kennard et al., 1967). In principle, crystal data could be obtained from poorly crystallized compounds with the aid of synchrotron radiation, but no data on glycoalkaloids have been published.

2.2 Starch in *Solanum* species

2.2.1 Tuber development

Cultivated potato (*S. tuberosum*) tubers contain mainly starch and water, as well as small amounts of fats, proteins and minerals. The tubers are mature when 97% of the final tuber size and starch content have been attained. Dry matter (DM) content of tubers is proportional to starch content, varying between 10 and 25 % of FW in mature tubers. Differences in starch deposition in the cells of pith and cortex can be observed in very young tubers depending on the variety, and these differences are relatively unaffected by environmental conditions. DM and starch content are low in early maturing cultivars and in immature tubers. After harvest, tubers have a resting period lasting 5-19 weeks when no sprouting occurs even under optimum conditions. The period of dormancy, when sprouting can be avoided, lasts approx. 18-33 weeks. After dormancy, starch starts to break down when the stored energy is needed for sprouting. Storage at low temperatures also causes starch to break down to small-molecule sugars (Cutter, 1992; Storey and Davies, 1992).

2.2.2 Structure

Starch structure has been studied intensively, and models have been developed to describe starch's structural levels from granule morphology to molecular structure. The model described in Figure 3 is well adapted for this purpose (Gallant et al., 1997). Storage parenchyma cells of potato tissue contain starch in the form of granules 15-100 μm in diameter. The morphology of starch granules has been studied by scanning electron microscopy (SEM) (Hall and Sayre, 1969; Hill and Dronzek, 1973; Barichello et al., 1990; Baldwin et al. 1994, 1997; Gallant et al., 1997; Tamaki et al., 1997; McPherson and Jane, 1999; Blennow et al., 2003; Singh et al., 2003;). Potato starch consists of linear amylose and branched amylopectin, which are polymers of glucose units joined by α-D-(1→4) glucosidic bonds, and amylopectin also with α-D-(1→6) bonds. Iodine-binding spectrophotometric studies have shown that proportion of amylose is typically 20-35%. The chain length distribution has been determined with MALDI-TOF MS and anion exchange chromatography with PAD (Broberg et al., 2000), and with size-exclusion chromatography (SEC) (Svegmark et al., 2002). In addition, starch granules contain 0.09% phosphorus in the forms of phosphate monoesters covalently bound to amylopectin and phospholipids complexed with amylose and long chains of amylopectin. The phosphorus content is affected by growing conditions, temperature and storage (Hoover, 2001).

X-ray scattering. X-ray methods have been applied to isolated potato starch since 1922 (Sponsler, 1922). Small-angle X-ray scattering (SAXS) is used to determine its structure at the level of 1-100 nm, e.g. nanostructure of starch. A long-range periodicity of 10 nm (100 Å) in moist starch was found using SAXS (Sterling, 1962). Subsequently it was proposed that starch consists of paracrystalline lamella embedded in an amorphous medium, with periodicity appearing at 9-10 nm (Cameron and Donald, 1992; Jenkins et al., 1993). Starch has also been described as a side-chain liquid-crystalline polymer, whose amylopectin double helices are twisted into a superhelix thus forming lamellae. The distance of 1.6 nm (16 Å) between the double helices is calculated from the position of the peak rising from the 100 reflection in the SAXS pattern (Waigh et al., 1998, 2000a). It has been possible to determine the crystallite size of 12-14 nm (120-140 Å) from the width of the 100 reflection, being the only well-separated peak (Jenkins and Donald, 1997).

The short-range structure of starch powder samples can be studied by wide-angle X-ray scattering (WAXS). The crystalline phase of starch is formed from the side chains of branched amylopectin double helices (Imberty et al. 1991; Jenkins et al. 1993). The crystalline structure has a hexagonal unit cell, with the lattice constants a = b = 18.3 Å and c = 10.5 Å, or a = b = 18.5 Å and c = 10.4 Å as determined from diffraction data (Imberty and Perez, 1988). The crystallinity, i.e. the portion of crystalline material, has been calculated from the diffraction profile of a starch sample. The crystallinity of approx. 24-28% for potato starch has been estimated by comparing the X-ray data of 70% crystalline cellulose (Nara et al., 1978; Zobel, 1988a). More recently, the crystallinity has been determined as a volume fraction of crystalline starch using Ruland's and Vonk's methods (Gernat et al., 1990). Relative crystallinity, e.g. how the crystallinity has changed as compared to native starch, has been determined in several studies using Wakelin's and Herman's methods (Jenkins and Donald, 1997; Mizuno et al., 1998; Paris et al., 1999; Waigh et al., 2000a).

The role of water. The distribution of water in starch granules has been studied using NMR techniques (Baianu et al., 1999; Tang et al., 2000). Water, an essential plasticizer in starch granules, pushes the helices into a lamellar structure (Waigh et al., 2000b). SAXS studies have shown that the water content of a starch sample has a great effect on the scattering intensities of the reflection 100 and on the intensities arising from the lamellar structure. Both reflections appear or disappear simultaneously with addition and removal of water, respectively (Perry and Donald, 2000). The effect of water content on native starch crystallinity has been determined by WAXS or solid-state NMR (Paris et al., 1999). However, the changes in the structure become weak when the hydration level is greater than 30% (w/w) (Nara et al., 1978; Imberty and Perez, 1988; Suzuki et al., 1997).

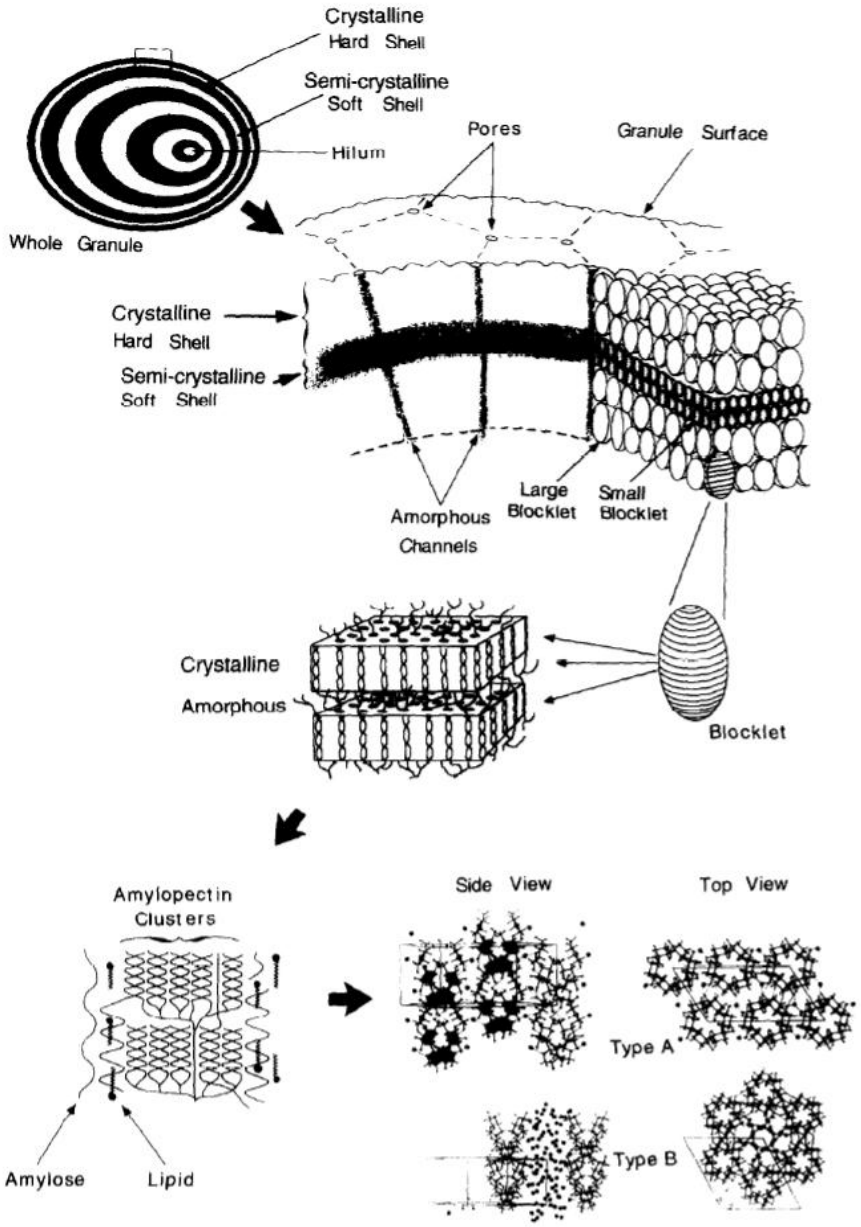


Figure 3. Starch granule structure. (Reprinted from Carbohydrate polymers, vol. 32, Gallant et al., Microscopy of starch: evidence of a new level of granule organization, page 188, 1997, with permission from Elsevier)

Changes due to temperature. Preparation of starch containing foods or potato processing often involves temperature changes that alter the starch structure. The gelatinization of starch takes place at elevated temperatures, e.g. in food preparation, when excess water molecules are linked to hydroxyl groups of amylose and amylopectin by hydrogen bonding. This gelatinization results in a breakdown of the double-helical order (Zobel, 1988b; Waigh et al., 2000a). The changes in starch structure due to phase transitions while heating or cooling have been examined with differential scanning calorimetry (DSC) and X-ray scattering. The crystalline state of starch, the amylose:amylopectin ratio, amylopectin branch chain length,

and water contents, affect the glass transition temperature (T_g) and melting temperatures (T_m), determined for starch by DSC (Mizuno et al., 1998; Jane et al., 1999; Tester et al., 1999). Phosphate in starch contributes to high viscosity and water-binding capacity as well as to low gelatinization temperature (Muhrbeck and Wischmann, 1998; Jane et al., 1999).

Effect of growth time and storage of tubers. Granule size increases with tuber weight. An increase in growth temperature has been shown to cause a decrease in the granule size but no changes in crystallinity were detected (Tester et al., 1999). However, starch crystalline structure determined by WAXS is similar in different cultivars, and no variations were observed during potato growth from day 42 to day 117 day (Sugimoto et al., 1995; Liu et al., 2003), or between different years (and weather conditions) (Svegmark et al., 2002).

2.2.3 Potato texture

Texture of boiled potato is classified experimentally in terms of disintegration, mouthfeel, and appearance. The texture of boiled potato is related to DM and starch content, starch granule size, degree of crystallinity, amylose:amylopectin ratio, cell size, and chemical composition of the tubers (Jarvis and Duncan, 1992; Van Soest et al., 1995; Van Marle et al., 1997; Baianu et al., 1999; Jane et al., 1999). No assumptions regarding potato texture should be made based only on the variety, because maturity, environmental, cultural, and storing conditions as well as potato size and preparation methods also affect the properties mentioned.

The gelatinization of starch involves swelling. Swelling, in turn, causes cell distension and separation of adjacent cells. Swelling during gelatinization exerts greater internal pressure in tubers with high DM or starch content, leading to a higher tendency for the potato to burst during boiling. Potato tubers with high disintegration are classified as 'mealy' (floury). Ripening increases the starch content and thus mealiness. Starch granule size is correlated with swelling power and water-binding capacity (Singh et al., 2003). For example, starch granule size was found to influence noodle quality (Chen et al., 2003). Furthermore, the crystallinity of starch is related to swelling properties (Zobel, 1988b; Mizuno et al., 1998; Tester et al., 1999) because gelatinization occurs primarily in the amorphous growth rings (Jenkins and Donald, 1997). As mentioned in the previous discussion, the influence of the crystallinity on the gelatinization and rheological properties of starch has not been thoroughly investigated (Hoover, 2001).

2.2.4 Effect of breeding on starch

The carbohydrate metabolism of potato can be affected by breeding and genetic modifications (Kortstee et al., 1998; Lloyd et al., 1999; Blennow et al., 2003; Dale et al., 2003). The ratio of amylose to amylopectin in starch can be regulated. A waxy potato starch with low amylose

content has been developed, and its different physicochemical properties have been examined (McPherson and Jane, 1999). Cultivars resistant to cold sweetening (sugar accumulation at low temperatures) have been developed with selection methods over a period of 12 to 15 years (Dale et al., 2003). No differences in starch granule size, total phosphorus content and gelatinization temperature of an interspecific hybrid between *S. tuberosum* and *S. chacoense* were detected compared to cultivated potato (Nowotna and Palasinski, 1986). However, higher stability of starch granules in DSC experiments was detected in a cold-sweetening resistant hybrid of between *S. tuberosum* and *S. phureja* (Leszkowiat et al., 1990). A higher degree of crystallinity of starch determined by WAXS was present in the hybrids compared to *S. tuberosum* (Barichello et al., 1990), but this finding has been questioned (Cottrell et al., 1995). No extensive data have been published on the starch structure, e.g. crystallinity, of tubers from interspecific hybrids.

3. AIM OF THE STUDY

When genome sections of wild *Solanum* species are bred into the cultivated potato to obtain improved potato cultivars, the new cultivars must be evaluated for their beneficial and undesirable traits. Glycoalkaloids present in *Solanum* species are known for their toxic effects on mammals. In addition to their toxicity, some glycoalkaloids present in the wild species also have beneficial properties. Starch content and composition affect the texture of cooked and processed potatoes. Because the crystalline properties are determined by the botanical source of the starch, the starch properties can be affected as a result of breeding.

The overall aim of the present thesis was to determine glycoalkaloid contents and starch nanostructure in cultivated (*Solanum tuberosum*) and wild *Solanum* potato species, with special attention to the effects of hybridization of *S. tuberosum* with related species on the nature and levels of glycoalkaloids as well as on starch nanostructures. The ultimate goal was to gain additional knowledge on the variability of glycoalkaloids and starch nanostructures in various interspecific potato hybrids that can be utilized as prebreeding cultivars and materials for the subsequent production of potato lines resistant to phytopathogens and environmental stress conditions.

To achieve the objectives, we carried out the following studies:

- High-performance liquid chromatography methods with diode array detection (HPLC-DAD), and electrospray ionization - mass spectrometry (HPLC-ESI-MS) for determination of glycoalkaloids and aglycones were optimized (Papers I, V)
- Solid-phase extraction (SPE) methods to prepare and clean glycoalkaloid extracts using different commercial sorbents were compared (Papers II, III, V)
- The HPLC-DAD method was applied to glycoalkaloid analysis of a wild *Solanum* species (Paper II), and the potato cultivars Satu and Sini (Paper III)
- The structures of glycoalkaloids in wild *Solanum* species and interspecific hybrids were determined, and the various glycoalkaloids were quantified by LC-ESI-MS (Paper V)
- X-ray scattering methods WAXS and SAXS were applied to fresh potato tuber samples for the first time. The samples were obtained from tubers at different maturities (Paper IV)
- The starch nanostructure of wild *Solanum* species and interspecific hybrids was studied using the WAXS and SAXS methods (Paper V)

4. EXPERIMENTAL

4.1 Glycoalkaloid analysis (I, II, III, V)

4.1.1 Analytical methods HPLC-DAD and LC-ESI-MS

The glycoalkaloids α-solanine (label purity ~95%), α-chaconine (label purity ~95%), and α-tomatine (label purity not given), and the aglycones solanidine, demissidine, tomatidine, and solasodine were obtained from Sigma (St. Louis, MO) and used as standard compounds. The compounds were dissolved in methanol. As demissidine showed very weak UV-absorption it was not included in LC-DAD studies. The proportion of dehydrotomatine in the commercial α-tomatine, and the proportion of dehydrotomatidine in commercial tomatidine were determined from their UV and MS spectra.

Reversed-phase high-performance liquid chromatography with diode-array detection (HPLC-DAD) was chosen for the separation of the glycoalkaloids and the aglycones (Paper I). The glycoalkaloids and the aglycones could be reliably detected at the wavelength of 205 nm. Different columns with C_{18} phases were compared in order to optimize the chromatography of the mixtures of the standard compounds. Acetonitrile with orthophosphoric acid (pH 3.0), triethyammonium phosphate buffer (TEAP) pH 3, triethylammonium acetate buffer (TEAA) pH 6.8-7.0, and Tris buffer pH 7.2, with ionic strengths of 25 mM, were used in elution testing. Column temperatures between 20 and 50°C were used. The chromatographic conditions and system information for the optimized methods are given in Table 4.

Mixtures of glycoalkaloids were also determined with reversed-phase liquid chromatography with quadrupole ion trap mass spectrometry equipped with an electrospray interface (LC-ESI-MS) (Paper V). A Luna C_{18}-2 (150 x 1 mm, Phenomenex Ltd.) chromatography column was used. The injection volume of the samples was 2 μl, containing ~0.1-2 μg of glycoalkaloids or aglycones. According to the results of previous studies with HPLC-DAD, acetonitrile with triethylammonium buffers was suitable for separation of the glycoalkaloids. However, the volatile mobile phase of acetonitrile-25 mM TEAA at pH 6.8 was used instead of TEAP buffer. The chromatographic conditions and system information are given in the Table 4.

The structures of the glycoalkaloids were determined by LC-ESI-MS according to *m/z* of protonated molecular ions $[M+H]^+$ and fragmentations of sugar moieties. It was possible to conduct multiple stages of MS with the aid of the ion trap. Quantification of glycoalkaloids was carried out using an external standard calibration method in the HPLC-DAD and in the LC-ESI-MS. In the HPLC-DAD, varying concentrations of α-solanine and α-chaconine standards were injected separately to obtain the calibration curves. The peak areas in these curves were then plotted against the concentration of each compound.

Table 4. HPLC methods used in glycoalkaloid analyses.

Method	System	Column (C_{18})	Mobile phases and elution conditions	Paper
HPLC-DAD[1]	Hewlett-Packard HP 1090A HPLC system (Waldbronn, Germany) with autoinjector, diode-array detector HP 1040A, HP 3392A integrator, HP 85B computer control and HP 9121D disk memory.	Zorbax-Rx, Zorbax-SB 250 mm x 4.6 mm 5 µm (Agilent Technologies).	Acetonitrile–TEAP buffer pH 3 (25mM) Linear gradient of 20–70% acetonitrile in 20 min or stepwise gradient of 20, 25, 35, 45 and 65% acetonitrile at time 0, 12, 15, 17 and 25 min. Flow rate 1-1.5 ml/min. Column 25-50 ºC	I, II
	Agilent Technologies 1100A HPLC-DAD (Waldbronn, Germany) with an autoinjector, and Chemstation software (release 08.03[847])	Zorbax-Rx 250 mm x 4.6 mm 5 µm (Agilent Technologies).	Acetonitrile–TEAP buffer pH 3 (25 mM) Stepwise gradient of 20, 25, 35, 45 and 65% acetonitrile at time 0, 12, 15, 17 and 25 min. Flow rate 1 ml/min. Column 50 ºC	III
LC-ESI-MS[2]	Agilent 1100 series HPLC-DAD, and HP ChemStation Plus A.07.01 software (Agilent Technologies, Palo Alto, CA). Mass spectrometric analysis: Esquire-LC QIT MS equipped with an electrospray interface (Bruker Daltonics, Bremen, Germany). The software Esquire-LC NT, version 3.1 (Bruker Daltonics).	Luna C_{18}-2 150 x 1 mm 5 µm (Phenomenex Ltd)	Acetonitrile-TEAA buffer pH 6.8 (25 mM) Linear gradient of 20 to 70% acetonitrile in 20 min, and that 70% was kept constant for another 55 min. Flow rate 50 µl/min. Column 50 ºC	V

[1] The effluent was monitored at 205 nm. On-line UV absorbance spectral data in the range of 190-400 nm were collected using the DAD system.

[2] Electrospray ionization was performed in positive ion mode at the scan range of *m/z* 100-1200. A capillary voltage of 2400 V and a trap drive value of 55 were used. The ICC was switched on. Multiple-stage MS(n) was carried out using helium as the collision gas.

In the LC-ESI-MS experiments, a mixture of standard compounds α-solanine, α-chaconine, and α-tomatine, or aglycones was prepared, and varying amounts were injected to LC-column.

4.1.2 Thin-layer chromatography (I)

Normal-phase TLC was performed on pre-coated silica gel 60 F254 sheets (Merck, Darmstadt, Germany). The TLC plates were developed in a chamber saturated with 95% ethanol or methanol-chloroform (2:1). The plates were sprayed with aqueous cobalt (II) thiocyanate solution or with sulphuric acid (95%)–methanol solution (1:1), and heated until the spots became visible. TLC was applied to glycoalkaloid standards, containing impurities (Paper I).

4.1.3 Preparation of the samples

The general procedure for glycoalkaloid extraction was as follows: Foliage materials were dried in the oven as described by Laurila (2005). Nonpeeled potato tubers were chopped and freeze-dried. Freeze-dried material and dried foliage were ground into fine powder with a mill. In addition, fresh nonpeeled tubers of the hybrid between *S. acaule* and *S. tuberosum* (2x *acl* + 4x *tbr*) were cut into pieces and extracted to study the effect of freeze-drying on the glycoalkaloid contents. Dried or fresh materials were homogenized in a 5% solution of acetic acid aided by Ultra-turrax. The suspension was centrifuged and the supernatant was filtered into a volume flask and the flask was filled with the solvent (Table 5).

Table 5. The ratio of the extraction solvent and plant materials in different experiments.

Sample material	Amount	Volume of 5% acetic acid	Sample/solvent ratio	Paper
Dried foliage	0.5 g	100 ml	1 g / 200 ml	V
Dried tubers	20 g	250 ml	1 g / 12.5 ml	V
	10 g	100 ml	1 g / 10 ml	III
Fresh tubers	50 g	250 ml	1 g / 5 ml	n

n = data not published in Paper I-V.

In order to develop a suitable solid-phase extraction (SPE) method for the clean-up of glycoalkaloid extracts, different SPE phases (octadecyl, octyl, benzene sulfonic acid, and macroporous copolymer) were applied to mixtures of standard compounds, and an extract of *brd* foliage (Paper II). An aliquot of the acetic acidic extract was applied to preconditioned SPE columns, and the undesirable components were washed with 5% aqueous methanol. The

glycoalkaloids were eluted with methanol or with 2.5% ammonia in methanol (for benzene sulfonic acid sorbents). The elution solvent was evaporated, and the residue was dissolved in methanol to obtain an LC sample. Recoveries of the glycoalkaloids and the aglycones from the applied SPEs were measured by spiking aliquots of the extracts with the standard compounds. The recoveries for the spiked glycoalkaloids and aglycones were calculated using the method recommended by IUPAC (Burns et al., 2002).

SPEs of the plant extracts were carried out for quantitative analyses of glycoalkaloids. As mentioned in the literature review, the most often used octadecyl phase is Sep-Pak C_{18} (Waters Corp., Milford, MA). This phase was selected for tuber extracts of cv. Satu and Sini analyzed using HPLC-DAD in the study reported in Paper III (Table 6). However, a silica-based strong cation exchanger (SCX), with a benzene sulfonic acid phase (Varian, Harbor City, CA) was selected for preparation of foliage and tuber samples for LC-ESI-MS measurements (Paper V), because it could be used successfully to clean the foliage extracts (Paper II). For purposes of comparison, an octadecyl phase (Sep-Pak C_{18}) was used simultaneously (new data). The other SPE methods reported in Paper II were not applied to other plant extracts in this thesis.

Table 6. Two SPE methods applied to quantitative analyses of glycoalkaloids in plant materials (methods described in Paper II are excluded).

Sorbent	***Plant material***	***Added compounds***	***Analytical method***	***Paper***
Sep-Pak C_{18}	*tbr* tubers	solanine, chaconine	HPLC-DAD	III
	tbr foliage	solanine, chaconine, tomatine demissidine, solasodine	LC-ESI-MS	n
SCX	*tbr* foliage	solanine, chaconine, tomatine demissidine, solasodine	LC-ESI-MS	V

n = data not published in Paper I-V.

4.1.4 Analysis of the plant extracts (II, III, V)

Varying plant materials were analyzed for their glycoalkaloids. The plant materials consisted of *S. tuberosum* (*tbr*) varieties as well as of wild species *S. brevidens* (*brd*), *S. acaule* (*acl*), and *S. commersonii* (*cmm*) and their interspecific hybrids. The plant materials of *tbr* as well as of wild species and somatic hybrids analyzed for glycoalkaloids are listed in Table 7. In addition to previously published results, the results from *cmm* and a male fertile symmetric somatic hybrid, SH9A, produced by electrofusion of mesophyll protoplasts between frost-tolerant *cmm* PI243503 and *tbr* SPV11 are included (Cardi et al. 1993a, b).

The optimized HPLC-DAD method (Zorbax-Rx C_{18} column and acetonitrile-TEAP buffer pH 3.0) (Table 4) was used for the analysis of α-tomatine and dehydrotomatine in *brd* foliage

(Paper II), as well as α-solanine and α-chaconine in tubers of the *tbr* cultivars Satu and Sini tubers (Paper III). It was necessary to use LC-ESI-MS in order to analyze the complex mixtures of glycoalkaloids present in *Solanum* species and interspecific hybrids. The plant extracts prepared with SPE methods were injected into LC columns and quantified using the external calibration method. In LC-ESI-MS experiments, all the spirosolanes were quantified as α-tomatine, and solanidanes as α-solanine, except for α-chaconine.

Table 7. Plant materials analyzed for glycoalkaloids using HPLC-DAD and/or LC-ESI-MS.

Plant material	**Method** [paper]	
	Foliage	***Tubers***
4x *tbr* Satu and Sini	-	DAD [III], MS [n]
4x *tbr* Pito	MS [V]	-
2x *tbr* White Lady 15	-	MS [V]
4x *acl*	MS [V]	MS [V]
2x *acl*+4x *tbr*	-	MS [V]
4x *acl*+2x *tbr*	MS [V]	-
2x *brd*	MS [V], DAD [II]	nontuberous
4x *brd*+2x *tbr*	MS [V]	-
1x *brd*+3x *tbr*	-	MS [V]
2x *cmm*	MS [n]	-
2x *cmm*+2x *tbr*	MS [n]	-

n = data not published in Paper I-V.

4.2 Starch nanostructure studies (IV, V)

4.2.1 Starch samples

Commercial starch powders (potato starch and modified starch Paselli WA-4 obtained from Avebe, The Netherlands) as well as potato tuber samples were used in this study. Commercial starch powders were pressed as pellets and measured using WAXS. Starch-water suspensions were covered with Mylar films (6.0 mm, Chemplex ind. Inc., NY, USA). The samples from raw tubers were either mashed or sliced tuber tissue (Papers IV, V). Potato slices with a thickness of ~1 mm were immediately measured to avoid drying. Three tubers were used for each determination. Distilled water was measured separately to model the diffraction of the water in fresh potato samples.

The potato tubers (cvs. Satu, Saturna, and Lady Rosetta) examined during winter storage were grown in the field and stored under controlled conditions at Boreal Plant Breeding (Jokioinen, Finland) (Paper IV). The tubers of wild species and interspecific hybrids for Paper V were produced, grown and stored at MTT Agrifood Research Finland (Jokioinen, Finland).

The tubers from various plant materials used for starch analysis with X-ray scattering methods are listed in Table 8.

Table 8. Plant materials analyzed for starch nanostructure with X-ray scattering.

Plant material	Paper
4x *tbr* Satu, Saturna, and Lady Rosetta	IV
2x *tbr* White Lady 15	V
4x *acl*	V
2x *acl*+4x *tbr*	V
2x *acl*+2x *tbr*	V
4x *acl*+2x *tbr*	V
2x *brd*+4x *tbr*	V
1x *brd*+3x *tbr*	V

4.2.2 Wide-angle X-ray scattering

WAXS experiments were carried out using symmetrical transmission geometry with Cu Kα radiation obtained from a sealed X-ray tube operated at 45 kV and 28 mA and monochromatized with a quartz monochromator in the incident beam. The scattered intensities were measured with a scintillation counter. For determination of the crystallinity and the lattice constants, the WAXS intensity was modelled by a linear combination of experimental intensities of (1) amorphous starch (modified starch Paselli WA-4) (2) water, and (3) a calculated diffraction pattern of crystalline component of starch. The model diffraction pattern of crystalline component was calculated as a sum of reflections of amylopectin. The experimental intensity curve was described in a model with a total of six parameters. The three nonlinear parameters were needed in order to calculate the diffraction pattern of amylopectin and the proportions of the intensities of water, amorphous starch and crystalline starch. The crystallinity of starch in the tuber sample was determined from the mass fractions of crystalline starch and of the sum of crystalline and amorphous phases. The detailed data analysis is described in Paper IV.

4.2.3 Small-angle X-ray scattering

SAXS measurements were made using a sealed fine-focus X-ray tube in the point-focus mode. Cu Kα radiation (wavelength = 1.54 Å) was monochromatized with a Ni filter and a totally reflecting mirror (Huber small-angle chamber 701), and the scattered intensity was measured with a Bruker AXS Hi-Star area detector. The intensities were corrected for absorption and nonsample scattering. The lamellar distance, the lattice constant *a* of the

hexagonal lattice of amylopectin, and the average size of the crystallites were determined by fitting a model in the SAXS intensity curve. The lamellar distance was determined from the position of the lamellar peak. The lattice constant *a* was determined from the *d* value of the reflection 100. The average crystallite size was determined from the full width at half maximum (FWHM) of the reflection 100 using the well-known Scherrer formula.

The USAXS experiments were carried out at Hamburger Synchrotronstrahlungslabor (HASYLAB, Germany) (Paper IV). The USAXS intensity curves showed the 9 nm lamella peak and two power-law like regimes in the smaller angles. The lamellar distance and the thickness of the lamella stacks were determined from the diffraction peak using a procedure similar to SAXS data analysis. The power law exponents were obtained.

5. RESULTS

5.1 Glycoalkaloid analysis

5.1.1 Purity of commercial α-tomatine and tomatidine (I, V)

On the basis of the HPLC-DAD and LC-ESI-MS experiments carried out in this study, the α-tomatine and tomatidine standard compounds contained dehydrotomatine and dehydrotomatidine, respectively. The purity of α-tomatine standard was 90-93%, as calculated from the peak areas of dehydrotomatine and α-tomatine obtained by MS detection. On the basis of the UV spectra, the purity of a tomatidine standard was 89-91% (data not shown). The calculation was based on results reported by Ono et al. (1997), where the extinction coefficient for α-tomatine was ~10 times higher than that of dehydrotomatine. The purity of 90% was used to quantify the spirosolanes in plant materials. The purity of tomatidine was estimated to be ~90%.

We also separated tomatidine and dehydrotomatidine on TLC plates. The color of spots visualized with the reagent and heat differed according to the aglycone structure The color with solasodine was purple and with tomatidine and dehydrotomatidine green. Commercial solasodine contained an additional unidentified component.

5.1.2 Analytical methods HPLC-DAD and LC-ESI-MS (I, V)

HPLC-DAD. Chromatographic separation conditions were optimized for simultaneous determination of glycoalkaloids and aglycones (Paper I). Acetonitrile with different aqueous buffers was tested for elution solvents. Retention time was decreased with orthophosphoric acid (pH 3.0) and acidic TEAP buffer (pH 3.0) as compared to separations with TEAA and Tris buffers at pH 7.0 and 7.4, respectively. Moreover, triethylammonium in the buffer contributed to better chromatography: the peaks of glycoalkaloids and aglycones narrowed (chromatograms not shown), and the retention times were repeatable. TEAA buffer resulted in a noisy baseline because of its high UV cut-off, and thus could not be used with UV detection at 205 nm.

When TEAP buffer was used, a 25-30% solution was necessary for proper separation of glycoalkaloids. A 40-60% acetonitrile solution was needed to separate the aglycones. The two different solvent gradients were employed for the simultaneous analysis of the glycoalkaloids and aglycones: a linear gradient from 20 to 70% acetonitrile in 20 min, or a stepwise gradient 20, 25, 35, 45, and 65% acetonitrile at times 0, 12, 15, 17, and 25 min. Approximately 40% lower retention times were achieved with the linear gradient. The separation of glycoalkaloids was improved with the stepwise gradient. Compared to separation at 20 °C (Paper I), column

temperatures of 40 or 50 °C caused a reduction in the retention times but with improved separation of the peaks.

Of the columns tested, Zorbax-Rx C_{18} and Zorbax-SB C_{18} eluted with acetonitrile-TEAP buffer (pH 3) gave the highest repeatability among the runs, and the best separation of the components. The main difference between Zorbax-Rx and Zorbax-SB was that Zorbax-SB separated the aglycones solasodine and tomatidine, whereas with Zorbax-Rx they were eluted in one peak. However, the baseline separation of all the glycoalkaloids was achieved only with Zorbax-Rx. Because the Zorbax-Rx column used with acetonitrile-TEAP buffer gave reproducible retention times, and thus this method was chosen for quantitative determination of α-solanine and α-chaconine in the cultivars Satu and Sini (Paper III).

LC-ESI-MS. Mixtures of glycoalkaloids were separated by HPLC, but the peaks for α-solanine, α-chaconine, α-tomatine, and dehydrotomatine partly overlapped. However, it was possible to integrate and quantify the compounds from extracted ion chromatograms (Figure 8 in Paper V). Direct injection of the sample into the MS and separation by the ion trap was inadequate, because glycoalkaloids with the same molecular masses emerged. Electrospray ionization produced protonated molecular ions $[M+H]^+$ from the glycoalkaloids present in the samples of the *Solanum* species and the interspecific hybrids. The $[M+H]^+$ ions were fragmented at multiple stages MS(n). Characteristic fragmentations were obtained from spirosolanes and solanidanes: spirosolanes were dehydrated at MS(2), yielding $[M+H-18]^+$ ion, and the saccharides were fragmented in the third stage of MS, whereas saccharides from solanidanes were fragmented already in the second stage. Glucose (Glc), galactose (Gal), rhamnose (Rha), and xylose (Xyl) present in the standard glycoalkaloids yielded neutral losses of *m/z* 162, 162, 146, and 132, respectively. The neutral loss series of *m/z* 132/162/162/162, obtained from compounds present in the plant extracts, was assumed to be lycotetraose. Similarly, it was assumed that the series of *m/z* 146/146/162 and 146/162/162 were chacotriose and solatriose, respectively. In additional studies, a commertetraose containing glycoalkaloids gave the series of *m/z* 162/162/162/162.

5.1.3 Preparation of the samples (II, III, V)

Extraction of glycoalkaloids from the dried plant material is preferred over extraction from fresh materials. In this study, all tuber materials were freeze-dried and the foliage materials were oven-dried. However, glycoalkaloids in *acl + tbr* tubers were determined from both fresh- and freeze-dried tubers by LC-ESI-MS. The average total concentration was virtually the same in the freeze-dried (15±0.7 mg/100 g FW) and fresh tubers (15±6.7 mg/100 g FW).

The recovery of spiked glycoalkaloids and the efficiency of clean-up were taken into account when SPE methods were compared for their suitability for glycoalkaloids. The highest recoveries were obtained with a macroporous copolymer sorbent (Oasis HLB, Waters) (Paper

II), which was used for glycoalkaloids by Alt et al. (2005). The Oasis HLB was not applied to any other plant materials in this study. The SCX sorbent gave the most efficient clean-up for foliage extracts, with good recoveries of 89-99% (Paper II). However, the recoveries were rather low for α-solanine (59%) and solasodine (43%) in the later experiment (Paper V) (Table 9).

As mentioned in the review, Sep-Pak C_{18} is the most frequently used SPE sorbent in glycoalkaloid analysis. In this study, reliable recoveries of 118% and 78% were obtained with Sep-Pak C_{18} for α-solanine and α-chaconine, respectively (Paper III). Sep-Pak C_{18} was applied also to a foliage extract of *tbr* analyzed using LC-ESI-MS, with reliable recoveries except for solasodine (Table 9).

Table 9. Recoveries±RSD of glycoalkaloids and aglycones from SCX and Sep-Pak C_{18} solid-phase extraction cartridges.

	Recovery (%)						
SPE sorbent	***α-Solanine***	***α-Chaconine***	***α-Tomatine***	***Demissidine***	***Solasodine***	**Method**	**Paper**
Sep-Pak C_{18} [a]	118±8.4	78±5.7	-	-	-	HPLC-DAD	III
Sep-Pak C_{18} [b]	111	94	83	67	36	LC-ESI-MS	n
SCX [c]	59±39	91±36	83±20	86±16	43±34	LC-ESI-MS	V

[a]Mean±RSD of two different spiking levels: 10 and 30 mg of a-solanine; 30 and 150 mg a-chaconine were added to a 5 mL aliquots of the extract of *tbr* cv. Sini (tubers) with three replications for each. [b]Mean of two replications: 100 µg of each compound were added to 5 mL aliquots of the extract of *tbr* cv. Pito (foliage). [c]Mean±RSD of three different spiking levels: 50, 100, and 150 µg of each compound were added to 5 mL aliquots of the extract of *tbr* cv. Pito (foliage) with two replications for each level. n = data not published in Paper I-V. - not determined.

5.1.4 Glycoalkaloid contents of the *Solanum* species and the hybrids

***Solanum tuberosum* (III, V).** The tubers of *tbr* cvs. Satu and Sini contained α-solanine and α-chaconine only. In HPLC-DAD chromatograms a minor peak appeared in front of α-solanine, but the peak could not be identified as a glycoalkaloid using LC-ESI-MS (data not shown). The total glycoalkaloid concentration of non-peeled and immature tubers was 9 to 11 mg/100 g FW (cv. Satu) and 23mg/100 g FW (cv. Sini) as determined by HPLC-DAD (Paper III). The ratio of α-solanine and α-chaconine was 0.19 and 0.36 in cv. Satu, and 0.50 in cv. Sini.

The tubers of dihaploid *tbr* White Lady 15. contained α-solanine and α-chaconine in the ratio of 0.34 determined by LC-ESI-MS. The concentration of 12 mg/100 g FW was at the level found in cultivated tetraploid varieties (Paper V). The concentration and glycoalkaloid profile of *tbr* foliage deviated from that of the tubers. The foliage of cv. Pito contained α-solanine and α-chaconine (810 mg/100 g FW), in a ratio of 0.62. In addition, solasonine or α-solamarine, and solamargine or β-solamarine were present (104 mg/100 g FW). Minor amounts of the compounds with *m/z* 850 and 866 were found in the *tbr* foliage. They were identified as dehydrosolanine and dehydrochaconine, both having dehydrosolanidine as the aglycone.

***Wild Solanum species* (II, V).** Glycoalkaloids α-tomatine and dehydrotomatine were separated and identified in *brd* foliage using HPLC-DAD. Glycoalkaloid profiles of the wild species *brd*, *acl*, and *cmm* were determined with LC-ESI-MS. The wild species contained no α-solanine and no α-chaconine typical for *tbr*, except for minor amounts in the foliage of *acl*. The glycoalkaloid profiles of the wild species (tubers and foliage) were rather simple, consisting of two or three major glycoalkaloids: α-tomatine and dehydrotomatine in *brd*; α-tomatine, demissine, and propably soladulcine B in *acl*. In this context, the exception was *cmm*, which contained ~63% of dehydrocommersonine, ~13% of commersonine, and ~14% seven different glycoalkaloids (Table 10).

The glycoalkaloid concentration of *acl* foliage was very low (26 mg/100 g FW), compared to that of foliage *tbr* (890 mg/100 g FW), *brd* (530 mg/100 g FW), and *cmm* foliage (110 mg/100 g FW). The tubers of *acl*, being very small in size, contained a high concentration of glycoalkaloids (48 mg/100 g FW).

Table 10. Glycoalkaloid contents of *cmm* and somatic hybrid foliage (mg / 100 g FW ± standard deviation) determined using LC-ESI-MS (data not published in Paper I-V).

$[M+H]^+$	Compound	Plant type	
		***cmm* CGN**	***cmm* + *tbr***
1032.8	Solasodine+lycotetraose	5.0±0.8	
	Dehydrotomatine	3.9±0.4	
1034.8	Soladulcine B	2.6±0.3	
	α-Tomatine	3.4±0.1	
886.7	β-Soladulcine	5.3±0.6	
1018.7	Demissine	4.8±1.3	
1016.7	Dehydrodemissine	5.1±0.5	
868.7	α-Solanine		281±14
852.8	α-Chaconine		399±18
1048.5	Commersonine	14±3.5	
1046.5	Dehydrocommersonine	69±15	4.4±0.3
	Total	110±23	685±35

Interspecific hybrids. Hybridization of the wild species and *tbr* resulted in complex mixtures of different glycoalkaloids (Figure 4). The foliage of 4x *brd* + 2x *tbr* contained α-tomatine as the main glycoalkaloid inherited from *brd*, and only minor amounts of α-solanine and α-chaconine. Tubers, however, contained mainly α-solanine and α-chaconine, 1x *brd* + 3x *tbr* with a larger proportion of *tbr* genome. The new combinations were found in tubers and foliage: (1) soladulcidine, or tomatidine bound to solatriose, or chacotriose, and (2) 5,6 - dihydrogenated form of solanidine, demissidine, bound to solatriose or chacotriose. In addition, demissine was found in *brd* + *tbr* hybrids. Demissine has not been found in the parental species *brd* or *tbr* but in many other *Solanum* species (Table 1). The main difference in the glycoalkaloid profiles between 4x *brd* + 2x *tbr* hybrids and 4x *acl* + 2x *tbr* hybrids was the larger proportion of demissidine, bound to solatriose or chacotriose, present in *acl* + *tbr*. The somatic hybrid *cmm* SH9A contained mainly α-solanine and α-chaconine, and a small amount of dehydrocommersonine.

The total glycoalkaloid concentrations of *brd* + *tbr*, and *acl* + *tbr* hybrid foliages were rather low, 88 mg and 180 mg / 100 g FW, respectively. However, the concentration of *acl* parental species was as low as 26 mg / 100 g FW in the foliage. As the glycoalkaloid profile became simpler in *cmm* hybrid, the total concentration increased fivefold.

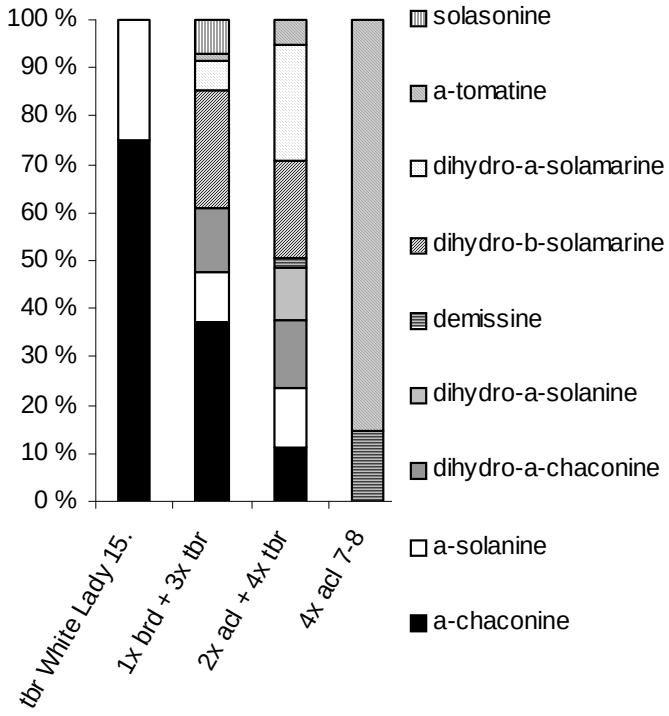


Figure 4. Proportions of different GAs detected in the tubers *tbr* White Lady 15. (*tbr*), the somatic hybrid of *brd* (*brd* + *tbr*), the somatic hybrid of *acl* (*acl* + *tbr*), and a parental species *acl* (*acl*). The total GA concentrations were 12±1.5, 13±2.6, 16±4.5, and 48±0.8 mg/100 g of FW, respectively.

Trace amounts of other compounds similar to glycoalkaloids also emerged in the foliage samples. In the hybrid between *brd* and *tbr*, the compound with *m*/*z* 864 consisted of chacotriose and an aglycone of *m*/*z* 410, but its structure could not be identified. The concentrations in the tubers remained under 20 mg / 100 g FW (13 mg and 16 mg /100 g FW in *brd* + *tbr* and *acl* + *tbr* hybrids, respectively). The ratio of α-solanine to α-chaconine in the tubers of the hybrids was 0.27 (*brd* + *tbr)*, and 0.63 (*acl* + *tbr)*, values typical also for *tbr* cultivars. The amounts of α-solanine and α-chaconine in the foliage were so low that their ratios were not determined.

5.2 Starch nanostructure

5.2.1 *Solanum tuberosum* (IV)

X-ray diffraction patterns obtained with WAXS from starch powder or tubers presented characteristic reflections of B-type starch structure (Figure 5). The lattice constants measured from fresh potato slices and mashes (varying between a = 18.4, c = 10.4 Å and a = 18.1, c = 10.5) were nearly identical to the values obtained with WAXS from starch–water suspensions (a = 18.3, c = 10.4). The dry starch samples had crystallinity between 12 and 16% with water contents between 7 and 13% determined from the diffraction data. Higher crystallinities were measured from starch–water suspensions. The crystallinity in potato slices and mashes was between 21 and 31% with water contents of 55–86%.

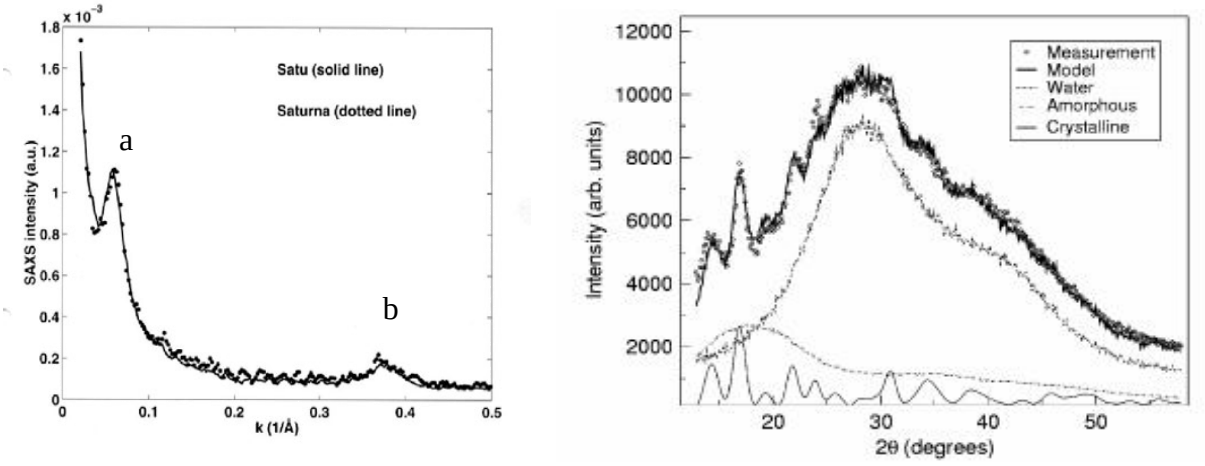


Figure 5. Left: SAXS intensity curves measured from fresh potato samples of the cvs. Satu and Saturna. (**a**) lamella peak (**b**) the 100 reflection corresponding to the interhelical distance. Right: WAXS diffraction patterns measured from a fresh potato sample, modeled by a linear combination of the experimental intensities of amorphous starch and water, and the calculated diffraction pattern of crystalline starch.

SAXS intensity curves of potato samples or moist starch included the reflection 100 and the peaks corresponding to the 9 nm lamellar structure (Figure 5). The lamellar distance was ~97 Å for the suspensions and 97±3Å for tubers. The thickness of lamella stacks was 513±6 Å. The values of the lattice constant *a* determined using SAXS from the reflection 100 were in a good agreement with those obtained using WAXS both for suspensions and potato flesh samples. The crystallite size in that direction was 127 Å in suspensions, and 98–143 Å in tubers.

The general feature of small angle scattering below the 9 nm peak was the presence of two power law regimes measured by USAXS. The lower angle regime was interpreted as scattering from rough surfaces, perhaps corresponding to the blocklet or superhelix structure of starch. The other power-law regime was typical for mass fractal structures, but the short range in which this power law was observed does not make this evidence very convincing.

As expected, the peaks shown by SAXS and USAXS were not found in any of the dry samples. The lamella peak appeared in SAXS curves when water was added to the dry powders. The USAXS intensities measured during the process of drying a potato slice showed that the structural change takes place in different stages. In the final stages of drying the lamellar structure collapsed fairly rapidly. The overall intensity of scattering continued to increase as the rest of the water evaporated, indicating that the intensity of the peaks rises from the voids.

5.2.2 Effect of winter storage (IV)

The starch nanostructures of three different cultivars of *tbr* were analyzed during one-year storage from August to May. No significant differences between the three cultivars were detected, except for the sliced samples measured in August. The average crystallinity of the potato samples was nearly constant (24%), whereas exceptionally high values were measured from sliced immature tubers in August: 41% for Satu, 50% for Saturna, and 34% for Lady Rosetta.

The lattice constants a = 18.4 and c = 10.4 Å for potato flesh samples were obtained from the year data measured between September and January (including cv. Satu, Saturna and Lady Rosetta, number of samples = 30, mashes and slices included). The means of the lattice constants obtained from potato slices in August and in May differed statistically significantly of the values measured between September and January. The change in the lattice constants did not follow the change in the water content determined from the WAXS data, the water content being over 55% in each sample. The crystallite size in the direction 100 or the lamellar distance determined with SAXS showed no significant differences between the slices and mashes or between the months. The means for the samples measured in August to May

were 118 Å for the crystallite size and 97 Å for the lamellar distance. The thickness of lamella stacks of 513 Å was measured in the slices only in September.

5.2.3 Wild species and interspecific hybrids (V)

The WAXS patterns showed that all tuber samples contained only B-type starch. Only the lattice constants *a* and *c* determined from the wild species *acl* deviated from *tbr* cultivars, and from the value obtained in interspecific hybrids, but the differences found were not statistically significant. Analyses of the cultivated potato, the wild potato species (*acl*), and the somatic hybrids (*acl + tbr, brd + tbr*) showed crystallinities between 22 and 29%, similar to the average crystallinity of 24% in *tbr* cultivars. The SAXS experiments revealed that the average size of starch crystallites calculated from the FWHM of the reflection 100 was comparable to that found for *acl*, interspecific hybrids and *tbr*. The lamellar distance, varying between 91 and 95 Å, was slightly lower than the value of 97 Å previously obtained for *tbr*, but the difference is not statistically significant. The thickness of the lamellar stack varied between 480 and 590 Å for all of the studied plant materials, which is in accordance with that for *tbr* cultivars, where a lamellar stack thickness of 513 Å was observed.

6. DISCUSSION

6.1 Glycoalkaloids

6.1.1 Analytical methods

Effective and reliable characterization of mixtures of glycoalkaloids present in plants was a time-consuming task prior to the development of modern analytical techniques, such as high performance liquid chromatography (HPLC), and gas-chromatography (GC), either alone or coupled to a mass spectrometer (LC-MS, GC-MS). A wide variety of different HPLC columns and elution solvents have been used for glycoalkaloid separations (Table 3), but the RP-HPLC-DAD method presented in this thesis is the only published method in which the four different glycoalkaloids and four different aglycones were separated simultaneously (Paper I). In addition, the Zorbax columns proved to be long-lasting. However, HPLC devices with columns of smaller size are now available and they should be used in order to minimize solvent volume and time used per analysis.

In this research, LC-ESI-MS was applied to *Solanum* plant samples (Paper V). It was possible to separate components in the different glycoalkaloid mixtures using chromatography. In addition, molecular masses of glycoalkaloids were determined from mass spectra. Multiple stages of MS were carried out with an ion trap. The structures of tri- and tetrasaccharides attached to aglycones were determined: neutral losses of *m/z* 162, 146, and 132 were assumed to be glucose or galactose, rhamnose, and xylose, respectively. It was possible to determine the molecular masses of the aglycones as well. For accurate structure determination, isolation and purification, followed by NMR-analysis, would be needed. However, the aim of this thesis was to obtain preliminary knowledge of the diversity of different glycoalkaloid structures in *Solanum* species and their interspecific hybrids. LC-ESI-MS proved to be a suitable tool for this purpose. For aglycone determination, a GC-MS method has been successfully developed (Laurila et al., 1996). LC-MS is preferable to GC methods, because no hydrolysis of glycosides is needed and both the structure of aglycone and the sugar moiety can be determined. RP-HPLC methods are suitable for α-solanine and α-chaconine determination, but the LC-MS was the only suitable method of determining several different glycoalkaloid structures in the plant materials considering the time and effort required. Quantification of glycoalkaloids with the LC-MS method is not as accurate as with HPLC-DAD because the intensities of the peaks in mass spectra are affected by the matrices, and the compounds in mixtures may affect ionization. However, the approximate levels of each glycoalkaloid in the plant materials were obtained.

Glycoalkaloids can be extracted reliably from the fresh materials, but freeze drying is the most frequently recommended method for plant sample pre-treatment (Dao and Friedman, 1996). In this thesis, SPE with C_{18} and SCX phases has been applied to glycoalkaloid extracts

with reliable recoveries (Paper II, III, V). According to the literature, SCX was applied to glycoalkaloid extracts for the first time in this thesis. Both C_{18} and SCX phases are suitable for cleaning up and concentrating glycoalkaloid extracts. However, cleaner samples were obtained from the foliage extracts with SCX.

To fulfil the quality requirements for potato as food, breeders have the responsibility to control the glycoalkaloid levels of new cultivars before and after releasing them to farmers and eventually to consumers. On the other hand, scientists are studying the structures of glycoalkaloids in order to understand their health-promoting effects. This, in turn, requires reliable analytical tools. Currently mainly α-solanine and α-chaconine levels of commercial cultivars are routinely being analyzed. Because glycoalkaloid profiles may change due to breeding methods or genetic modifications (GM), analytical methods such as GC-MS, RP-HPLC, or LC-MS should be used in tests on new cultivars in control laboratories.

6.1.2 *Solanum tuberosum*

In this study, the total glycoalkaloid content of immature tubers was at an acceptable level (under 20 mg/100 g of FW) in the cv. Satu and in tubers of diploid cv. White Lady, both grown in the greenhouse. The concentration of glycoalkaloids in mature Satu tubers is typically 4 to 6 mg/100 g FW (Pietilä, 2000). The high concentrations of glycoalkaloids typical for cv. Sini (Blomberg and Penttilä, 2003) were also detected in this study. The ratio of α-solanine and α-chaconine varied between 0.19 and 0.50. According the values reviewd by Friedman (2003), the value of 0.19 detected in Satu is lower than the value reported by other investigators, usually above 0.3. Because α-chaconine is more toxic than α-solanine, this result indicates that the ratio in Satu was not favourable from safety considerations. However, the ratio may vary depending on growing conditions and year of harvest. In the latter experiment, the ratio in Satu was 0.30.

Tubers of *tbr* contained only α-solanine and α-chaconine, but the foliage of cv. Pito contained spirosolanes as well, accounting for ~12% of the total glycoalkaloids. Minor amounts of spirosolanes in the foliage and in aged as well as in wounded tubers have been reported (Shih and Kue, 1974; Sinden and Sanford, 1981; Chivanov et al., 2001). The results indicate that the spirosolanes tend to be formed under stress.

The glycoalkaloid concentration of foliage of cv. Pito was 890 mg/100 g FW as determined with LC-ESI-MS. Lower amounts of ~50-220 mg/100 g FW in leaves and stems have been reported (Laurila, 2004; Friedman, 2006). Such results indicate that the plants of cv. Pito could have been suffering from stress. The compounds dehydrosolanine and dehydrochaconine in the *tbr* foliage samples detected in this study have also been detected in transgenic potato tubers (Bianco et al. 2003; Stobiecki et al 2003).

The glycoalkaloid levels in tubers of registered cultivars are usually at the acceptable levels. Unfortunately, potatoes sold in bulk are often exposed to light, causing greening and mechanical damage during storage. This may cause glycoalkaloid levels in tubers to rise, resulting in acute symptoms, such as gastrointestinal disorders in consumers. Furthermore, the recommended level of 20 mg/100 g FW is under debate because the synergistic effects of glycoalkaloids are not yet fully understood (Hopkins, 1995; Korpan et al. 2004; Friedman, 2006). A maximum level of 10 mg/100 g FW has been suggested by the Nordic countries in order to assure the safety of potatoes as food (Blomberg and Hallikainen, 2000).

6.1.3 Wild species

The total concentrations of glycoalkaloids in the foliages varied widely depending on the species, the concentration decreasing in the order *tbr* > *brd* > *cmm* > *acl* (Table 2 in Paper V, corrected version). The glycoalkaloid profiles of the wild species were completely different from those of *tbr*. The results obtained in glycoalkaloid analysis of the wild *Solanum* species agreed in part with those of previous research (Table 1). An exception was *acl* extract, which caused two different peaks in the chromatogram, both components showing mass fragments fitting to a structure of α-tomatine. The structure was assumed to be that of soladulcine B. α-tomatine and soladulcine B have different aglycones that are diastereoisomers of each other. Soladulcine B has not previously been detected in *acl*. More studies of this component are needed to confirm this result. In addition, demissine was detected in *acl*, as reported in other papers (Osman et al., 1978; Gregory et al., 1981; Kozukue et al., 1999; Rokka et al., 2005). *Solanum acaule* expresses desirable traits: resistance to diseases and potato viruses, as well as tolerance to frost and cold. This thesis did not attempt to assess the significance of glycoalkaloid contents for the plant's defence system. However, aglycone accumulation appears to decrease in response to soft rot in *acl* (Rokka et al., 2005).

Solanum brevidens contained significant amounts of α-tomatine, as also observed previously by Deahl (1993). The structures of aglycones tomatidine and dehydrotomatidine in *brd* were determined by Laurila et al. (1999). In this study, the presence of dehydrotomatine in *brd* was ascertained for the first time (paper II, V). The results were based on the LC-ESI-MS measurement of commercial α-tomatine containing dehydrotomatine and in the the plant samples. The fact that both dehydrotomatine and α-tomatine also appear in tomato plants (Friedman and Levin, 1998) has promted researchers to raise the question as to why a plant, such as tomato, synthesizes both of these glycoalkaloids, and whether they act synergistically as do α-solanine and α-chaconine found in potatoes (Friedman, 2002). Although these subjects were not addressed here, the new finding that dehydrotomatine and α-tomatine are present in *brd* extends the knowledge of glycoalkaloid structures: they are created in pairs in the plant. Whether the glycoalkaloid content is connected to several desired traits emerging in *brd*, such as resistance to varying potato viruses, has not been investigated. However, α-

tomatine level in tomato has been correlated with fungal resistance. However, conflicting results have been obtained regarding resistance to Colorado potato beetles (CPB), as thoroughly reviewed by Friedman (2002).

The species *cmm* had the most diverse mixture of glycoalkaloids evaluated in the present study. Of the detected glycoalkaloids, solasodine bound to lycotetraose, soladulcidine bound to lycotetraose (Soladulcine B), and soladulcidine bound to solatriose (β-Soladulcine) have not been previously characterized in *cmm*. Carputo et al. (2003) reported only the presence of dehydrotomatine, dehydrodemissine, and dehydrocommersonine in tetraploid *cmm* (PI243503). However, other studies showed the presence of demissine, commersonine, dehydrodemissine, dehydrocommersonine, and α-tomatine in diploid *cmm* (Vazquez et al., 1997). Despite the variation in the *cmm* profiles, *cmm* typically seems to contain lycotetraose or commertetraose sugar moieties, mainly bound to solanidine or demissidine. While high commersonine or dehydrocommersonine contents reduced feeding by CPB in *S. chacoense* (Sinden et al., 1980), such resistance in *cmm* has not been previouly reported. *S. commersonii* is noted mainly for its high frost tolerance.

6.1.4 Interspecific hybrids

As new cultivars are being developed using wild germplasm, e.g. in interspecific hybridization, glycoalkaloid profiles of foliage and tubers are being altered. The results of this research showed that complex mixtures of glycoalkaloids were formed in interspecific hybrids. Glycoalkaloids of both parental plants as well as new combinations of aglycones and saccharides were detected. The new combinations emerging in tubers and foliage of *acl* + *tbr*, and *brd* + *tbr* were solatriose- or chacotriose-containing spirosolanes, aglycones being either soladulcidine or tomatidine (diastereoisomers). Demissidine, bound to solatriose or chacotriose, was detected especially in the hybrids of *acl* + *tbr*. These new combinations have not been reported previously either in any *Solanum* plants, section *Petota* (Table 1), or in hybrids.

In addition, demissine, which is not found in parental species *brd* or *tbr* but in *acl* and many other *Solanum* species, was found in *brd* + *tbr* hybrids. The hypothesis has been proposed for the mechanism by which demissidine aglycone is formed from parental solanidine in hybridization (Laurila et al., 1996). The main difference in the glycoalkaloid profiles between 4x *brd* + 2x *tbr* hybrids and 4x *acl* + 2x *tbr* hybrids was the larger proportion of demissidine, bound to solatriose or chacotriose, present in *acl* + *tbr*. The hybrids *acl* + *tbr* and *brd* + *tbr* contained mainly glycoalkaloid structures having no 5,6-double bond in the aglycone. This could be considered a positive trait because of the lower toxicity of such glycoalkaloids compared to 5,6-unsaturated molecules. *Solanum tuberosum* cultivars contain mainly solanidine glycoalkaloids, which have the 5, 6-double bond.

The somatic hybrid *cmm* + *tbr* SH9A contained mainly α-solanine and α-chaconine, as well as a small amount of dehydrocommersonine. This result is in accordance with findings reported by Esposito et al. (2002), who detected the same glycoalkaloids in the SH9A hybrid tubers.

Because the hybrids examined in this thesis are not cultivars used commercially, the glycoalkaloid content of new cultivars cannot be directly predicted from the results of these analyses. However, Laurila (2004) found that backcrossing to *tbr* reduced the proportion of the "alien" aglycones. However, minor amounts of "alien glycoalkaloids" were still present. On the positive side, the spirosolane glycoalkaloids that emerge in the backcrosses may have beneficial effects in human health (Friedman, 2002). It is, therefore, possible that hybridization with *acl*, *brd*, *S. berthaultii*, and *S. circaefolium* would impart beneficial changes in the glycoalkaloid profiles (Table 2). However, evaluation of the total activities of glycoalkaloid mixtures in ingested potatoes is difficult, because combinations of glycoalkaloids act synergistically, additively, or antagonistically.

Previous research has called attention to significant differences in the glycoalkaloid profiles between interspecific hybrids and genetically modified potatoes. Genetic modification (GM) via gene transfer caused no significant changes in the glycoalkaloid profiles of potatoes (Rogan et al., 2000; Bianco et al., 2003; Stobiecki et al., 2003; Zuk et al., 2003; Matthews et al., 2005). The results presented in this thesis as well as several published studies suggest that the formation of new glycoalkaloids in potatoes is achieved through hybridization.

Glycoalkaloids, e.g. solasodine, are used in the pharmaceutical industry as starting materials in hormone synthesis. Furthermore, isolated glycoalkaloids, such as α-chaconine, have medical applications (Lee et al., 2004). Because the sugar moiety affects the activity of a molecule, the enzymatical method for *trans*-glycosylation of lycotetraose (present in α-tomatine) and cholesterol has been developed (Ikeda et al., 2006). Research is needed to determine whether this kind of targeted synthesis is also possible in modified plants. Generating potato plants that create new health-promoting molecules would be an interesting challenge for plant scientists that may benefit food safety and human health.

6.2 Starch

6.2.1 *Solanum tuberosum*

X-ray scattering measurements have commonly been conducted on isolated starch powders. In the present study however, starch in potato tubers was studied by measuring fresh cuts of tubers (Paper IV). As the samples contained a large amount of water, scattering data of pure water were measured and used in data modelling. The parameters calculated from the cuts were in accordance with the literature values obtained from isolated starch. The lattice

constants varied between *a* = 18.4 Å, *c* = 10.4 Å and *a* = 18.1 Å, *c* = 10.5Å, which were close to *a* = 18.3 Å, *c* = 10.5 Å or *a* = 18.5Å, *c* = 10.4 Å as determined from isolated starches by other authors (rev. in Imberty and Perez, 1988). The results confirmed that the X-ray scattering of starch can be carried out without isolating the starch from tubers. This is advantageous when large amounts of different tuber samples are to be analyzed. On the other hand, samples for X-ray measurements can be prepared from very small tubers with a diameter of ~1 cm. Furthermore, different levels of starch structure were determined: the proportion of the crystalline material in the sample (crystallinity) and the average crystallite size. In addition, the distance of periodicity in alternating layers of amorphous and crystalline material were determined.

The crystallinity of starch in potato slices and mashes varied between 21 and 31%. This result is in accordance with the literature values for potato starches (Hoover, 2001). Furthermore, the average crystallite size of ~130 Å, and the lamellar distance of ~100 Å in potato cuts were similar to values measured in isolated starch (Cameron and Donald, 1992; Jenkins et al., 1993; Jenkins and Donald, 1997).

The nanostructure of starch in cv. Satu, Saturna, and Lady Rosetta was studied monthly during winter storage of tubers. No differences between the cultivars were detected. However, exceptionally high crystallinity of the samples was detected in immature tubers (before harvest). Starch content is low in immature tubers, and starch synthesis is still in progress. High crystallinity may be due to short chain sachharides, which can arrange themselves in a more orderly fashion than the long chain starch molecules. The differences in starch structures were detected also in the change of lattice constants in August.

6.2.2 Wild species and interspecific hybrids

In the present study, the starch nanostructure in the samples of *acl* and in the interspecific *acl* + *tbr* and *brd* + *tbr* hybrids determined by X-ray scattering was reported for the first time. The samples consisted of B-type starch, such as the starch formed in the cultivated varieties of *tbr*. All the parameters calculated from WAXS and SAXS patterns of the hybrids, were similar to those of cultivated varieties. This indicates that no changes in starch nanostructure occur as a result of hybridization. Deposition of starch in *Solanum* plants and tuber formation is dependent on genetic background, as *Solanum* species include tuber-bearing as well as non-tuberizing species, e.g. *brd*. Carbohydrate metabolism of *brd* (Bánfalvi et al., 1999) and tuberizing *S. demissum* (Helder and Vreugdenhil, 1999) have been investigated, but the starch structure and properties of wild species have not been examined extensively, probably because the starch of wild species has no industrial use.

7. CONCLUSIONS

In the present study, separation of glycoalkaloids and aglycones was improved using reversed-phase high-performance liquid chromatography equipped with a high-quality silica-based octadecyl chromatography column, using acetonitrile-acidic triethylammoniumphosphate buffer as an elution solvent. This method can be used for simultaneous determination of glycoalkaloids and aglycones. The HPLC method was successfully applied to the quantitative determination of α-solanine and α-chaconine in cultivated potato tubers. In addition, clean-up of foliage samples was improved using a strong cation exchange resin instead of octadecyl phases in solid-phase extraction.

Solanum species and interspecific somatic hybrids were analyzed for their glycoalkaloid contents using liquid chromatography-electrospray ionization-mass spectrometry. Both tuber and foliage samples were included in the analyses. The elucididation of the molecular structures of glycoalkaloids was based on multiple stages mass spectral fragmentation of the components. The LC-ESI-MS method proved to be well-suited to the analysis of complex mixtures of glycoalkaloids. The method is recommended for screening new glycoalkaloid structures or "alien" glycoalkaloids emerging in potatoes due to breeding.

The following glycoalkaloids were detected for the first time in the plants: soladulcine B in *acl*, dehydrotomatine in *brd*, as well as solasodine bound to lycotetraose, Soladulcine B, and β-Soladulcine in *cmm*. In addition to parental glycoalkaloids, new combinations of aglycones and saccharides were detected in the interspecific somatic hybrids. The new combinations emerging in the tubers and in the foliage of *acl* + *tbr*, and *brd* + *tbr* were soladulcidine or tomatidine bound to solatriose or chacotriose. Demissidine bound to solatriose or chacotriose was detected especially in the hybrids of *acl* + *tbr*. Any of these new combinations have not been previously reported in *Solanum* species, section *Petota*. Furthermore, the combinations have not previously been detected in any hybrids. Demissine not found in parental species *brd* or *tbr* but in *acl* and many other *Solanum* species, was found in *brd* + *tbr* hybrids.

Based on these results, the glycoalkaloid profiles of the hybrids may represent a safer and more beneficial spectrum of glycoalkaloids than that found in cultivated varieties of *tbr*. The hybrids contained mainly spirosolanes, and glycoalkaloid structures having no 5,6-double bond in the aglycone. However, evaluation of the total behavior of glycoalkaloid mixtures in potatoes consumed is difficult, because the glycoalkaloids act synergistically. New glycoalkaloids as well as mixtures of glycoalkaloids should be tested, and their biological activity evaluated, so that potentially health-promoting or safe hybrids will be used in breeding.

Starch nanostructure of *tbr* cultivars, *acl* and interspecific hybrids were examined by wide-angle and small-angle X-ray scattering. For the first time, the measurements were conducted

on fresh potato tuber samples. Crystallinity of starch, average crystallite size, and lamellar distance was determined from the X-ray patterns. Using WAXS and SAXS to study starch nanostructure in samples from tuber slices can be recommended, especially when comparisons between large numbers of different samples are made, because this eliminates the need to isolate the starch from the tubers.

It was not possible to relate the different processing properties of the cultivars Satu, Saturna, and Lady Rosetta to the results obtained by WAXS and SAXS, because no differences in the starch nanostructure between the cultivars were detected. However, tuber immaturity was detected by X-ray scattering methods when large numbers of immature and mature samples were measured and the results were compared. No changes in starch nanostructure were observed as a result of hybridization. In contrast to results derived from genetic modification of potatoes by gene transfers, the the present study shows that no significant changes occurred in the nanostructures of starches resulting from hybridizations of potato cultivars.

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